Life history trade-offs, immune function and the expression of sexual signals in two model groups of birds (Psittaciformes, Charadriiformes).

by

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Abstract

Ecological immunology is the study of the ecological and evolutionary factors that explain variation in the function of, and investment in, the immune system. Within this field, reproduction-based trade-offs are an important focus, where studies often address the immunological costs associated with the expression of secondary sexual traits used in mate choice. Specifically, the Immunocompetence Handicap Hypothesis (ICHH) links the expression of secondary sexual traits to immune function, stating that testosterone promotes the expression of these traits while suppressing the immune system. In doing so, testosterone may maintain the honest expression of such traits, but also ultimately cause sex differences in immunity because males tend to have high levels of testosterone. I explore aspects of these topics using two model systems: the Psittaciformes (Parrots: Chapter 2) and the Charadriiformes (shorebirds), in particular the sex-role reversed Red phalarope (*Phalaropus fulicarius*). In Chapter 2, I employed a phylogenetically informed approach to investigate the relationships among immune investment, plumage colouration and longevity in parrots. I found that immune investment was greater in more colourful species, as well as in those with a slower pace-of-life (i.e., longer incubation periods), but not specifically in those with longer lifespans. In Chapter 3, I investigated the role that reproductive behaviours play in determining sex differences in corticosterone levels. One explanation for sex differences in the stress response is that selection favours a reduced response in incubating birds to reduce nest abandonment. I generally found little support for sex differences in corticosterone being driven by behaviours related to incubation. Moreover, in phalaropes, sex differences in corticosterone were already present prior to incubation. In Chapter 4, I found that males have higher levels of testosterone than females, but that females were immunosuppressed compared to males. However, I found evidence that testosterone may regulate immune function in females, but not males. The observation of female-biased immunosuppression is consistent with Bateman's Principle, and although there was some evidence of a testosterone-mediated handicap acting through immune function, these results attest to a fundamental lability in the relationship between testosterone and immunity. In Chapter 5, I demonstrated that plumage colouration in phalaropes is condition-dependent and so potentially conveys useful information to conspecifics. Yet, the relationship was negative in both sexes such that more colourful individuals had poorer immunocompetence, which was contrary to predictions. In Chapter 6, I demonstrated that phalaropes pair assortatively (positively) based on plumage colouration, but negatively based on aspects of size. I discuss the results of this dissertation in the light of life history theory, as well as in the context of mechanisms maintaining signal honesty.

Acknowledgements

I guess I would like to start by apologizing for not having a more inspired acknowledgements section. But I have diligently and enthusiastically recorded the names of everyone who has contributed to, even lightly brushed up against, this dissertation since I started.

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Prologue

I began my 'academic career' as what I would call a herpetologist. Let's face it, the group has a pretty universal appeal. During my M.Sc. came a (r)evolutionary change on two fronts. Firstly, my project on salamanders was put aside after some [demonic] growing pains, and the hole that it left was filled with birds since I had experience with them, too. Birds were deemed 'safer'. The other thing that happened was Mark Mallory, a seabird biologist with the Canadian Wildlife Service, joined the lab and through him I started working in the arctic (and met Grant). Through the transition from herps to birds, the one constancy was the type of questions...largely evolutionary based, and not entirely taxon specific.

I think it was pretty early in my arctic experience, sitting at the top of foggy cliffs at Cape Vera, fussing over an uncooperative radio receiver (or solar panel array, or <insert whatever you like since most things stopped working at one point or another>) with zero prospects of fixing it that field season, I audibly said to myself "there is no way I am doing any of my personal research in the arctic...this is ridiculous!" Fieldwork is complicated enough, and the arctic takes it to a whole new layer. The fact is why would I work in the arctic if I could choose another system to ask my taxon non-specific questions? Perhaps a warm place, where the birds breed all year long! Sometime afterwards it occurred to me, a great model to test theories in sexual selection, and in particular signalling theory, is one that bucks several significant trends....a sex-role reversed species. And where do you find sex-role reversed species? Well, several places actually but the arctic is a hot spot. And so back I went.....(but bear with me, I begin the story with parrots.)

List of Original Papers

The chapters of this thesis represent manuscripts outlined below.

Chapter 2:
Edwards, D.B. 2012. Immune investment is explained by sexual selection and pace-of-life, but not longevity in parrots (Psittaciformes). PLoS ONE 7: e53066.

Chapter 3:
Edwards, D.B., E.H. Chin, G. Burness, A.I. Schulte-Hostedde and H.G. Gilchrist. 2013. Linking sex differences in corticosterone with individual reproductive behaviour and hatch success in two species of uniparental shorebirds. Comparative Biochemistry and Physiology A 166:169-176. doi:10.1016/j.cbpa.2013.05.024

Chapter 4:
Edwards, D.B., G. Burness, A.I. Schulte-Hostedde and H.G. Gilchrist Sex-biased immunocompetence in a sex-role reversed bird. *Manuscript*

Chapter 5:
Edwards, D.B., S.M. Doucet, H.G. Gilchrist and A.I. Schulte-Hostedde. Condition and testosterone dependent plumage colouration and sexual dichromatism in a sex-role reversed bird, the Red phalarope. *Manuscript*

Chapter 6:
Edwards, D.B., H.G. Gilchrist and A.I. Schulte-Hostedde. On the fringe of mutual mate choice: positive plumage, and negative size, assortative mating in a polyandrous shorebird. *Manuscript*

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CHAPTER 1: General Introduction

"Life history theory predicts how natural selection should shape the way organisms parcel their resources into making babies" (Reznick 2010).

Life History Theory/Trade-offs between Reproduction and Survival

Since Roff (1992) and Stearns (1992) formalized life history theory, it has become a pervasive concept in the study of just about every aspect of ecology and evolution. Life history theory describes how groups of traits combine to increase reproductive success as a response to selection, but do so as a function of trade-offs amongst one another due to time, resource or physiological constraints. Life history theory seeks to explain the optimal allocation of resources to growth, reproduction and survival in the face of ecological challenges.

Natural selection should produce life histories that maximize lifetime reproductive success. For some species, maximizing reproductive success results from high rates of reproduction with limited parental care, while in others this is achieved by low rates of annual reproduction and a high degree of parental care (Roff 1992). This variation in life histories is often referred to as pace-of-life (POL) because other traits such as faster rates of growth, shorter times to reach sexual maturity and reduced longevity are typically coupled with high rates of reproduction (i.e., short but intense bouts of breeding, large clutches). Such species, those with short lifespans and high annual investment in reproduction, are said to lead fast-paced lives; fast-paced species are characterized as investing in current, rather than future reproduction (i.e., survival).

Bateman (1948) was the first to articulate that males and females differ in their investment in current versus future reproduction. Typically, males can maximize reproductive success through high rates of current reproduction whereas females do so by investing in

survival, and therefore future reproduction. Among mammals, males generally have a higher rate of mortality than females (Moore and Wilson 2002) and this difference is thought to be directly attributable to the strength of sexual selection facing males. In extreme cases, competition among males for mating opportunities can result in semelparity in males, but not in females (Boonstra *et al*. 2001). In birds, male mortality also increases with the strength of sexual selection (Liker and Székely 2004). Moreover, adult sex ratio in birds is related to social mating system such that, for example, polygynous species tend to have female-biased adult sex ratios (and vice versa for polyandrous species; Liker *et al*. 2013). Given that the sex ratio at birth tends to be 1:1 (Benito and Gonzalez-Solis 2007), the assumption is that there is higher post-hatch mortality in the sex facing high levels of sexual selection.

Ecological Immunology

Trade-offs among immune function and other life history traits, chief among them reproduction, are likely important determinants of life history evolution (Sheldon and Verhulst 1996, Schulenberg *et al*. 2009, Martin *et al*. 2011). Our understanding of life history trade-offs is predicated on the premise that resources are finite and that reproduction and immune functions are costly. While there is ample evidence of the former (e.g., Roff 1992, Rose and Bradley 1998, Harshman and Zera 2007), there is debate about how or whether immune function is traded-off against other life history traits (Hasselquist and Nilsson 2012).

Theoretically, immunity is costly. Components of the immune system must be synthesized, which requires energy for their production as well as physical resources (e.g., protein). Innate components such as leukocytes require constant synthesis to maintain baseline levels. For acquired components, such as antibodies, synthesis requires acute up-regulation in response to an immune challenge (Klasing 2004). For example, in endotherms fever often

accompanies an immune response and the increase in metabolic rate to generate an increase in body temperature likely consumes considerable energetic resources (Kluger *et al*. 1998). However, the immune system itself may require many fewer resources than other processes, such as growth (Klasing 1998).

In practice, the energetic costs associated with immune function are complex. For example, birds mount a weaker immune response under restricted food regimes (e.g., Brzek and Konarzewski 2007, Gutierrez *et al*. 2011) demonstrating that a lack of resources can limit the degree of response to pathogens. Similarly, an immune challenge can result in a reduction in body mass presumably because the response uses intrinsic energy stores (e.g., Bonneaud *et al*. 2003, Bonneaud *et al*. 2012). Activation of the immune system can also cause an increase in metabolic rate (e.g., Martin *et al*. 2003, Burness *et al*. 2010), suggesting an energetic cost. However, whether immune function is actually energetically constrained has been called into question in light of numerous studies that have failed to support all or some major predictions of a resource-based trade-off involving the immune system (e.g., Burness *et al*. 2010, King and Swanson 2013; also reviewed in Hasselquist and Nilsson 2012). The equivocal nature of these studies is likely a result of the complexity of the immune system, and the numerous ways of assessing costliness.

Abad-Gomez *et al*. (2013) present a useful case study illustrating the complexity of energy-based trade-offs involving the immune system. The authors injected plovers (*Charadrius dubius*) with a novel antigen to stimulate a humoral immune response. During the initial exposure, the plovers produced relatively low numbers of antibodies in response to the antigen (primary response). Following a second injection, the plovers mounted a much stronger response due to immunological memory (secondary response). The plovers did not significantly increase

their resting metabolic rate during the primary response, but did so during the secondary response. Yet birds did lose mass during the primary response. These results, and the fact that peaks in antibody production did not precisely correspond with elevated metabolic rate, highlight a discrepancy between energy vs. resource use, and how resources are used during an immune response. Thus, this case study helps to illustrate the complexity of the immune system and the challenges associated with understanding the interplay between immune function and energy-based trade-offs.

Trade-offs between the Immune System and Reproduction

Following the principles of life history theory and ecological immunology, investing more in reproduction should come at the expense of immune function (Sheldon and Verhulst 1996, Zuk and Stoehr 2002). There are several lines of evidence supporting this notion.

a) Proximate Energy-based Trade-offs

Studies that demonstrate energy-based trade-offs at the level of the individual are numerous. For example, manipulating clutch size of birds can result in increased parasite loads and reduced immune function in those females with enlarged clutches (Knowles *et al*. 2009). Experimentally enlarged clutches increase the workload for the parents and therefore places a greater demand on resources required for other processes, such as immunity. Furthermore, injection of a novel antigen can decrease reproductive success by prolonging incubation (Hanssen 2006) or reducing the rate at which young are fed (Råberg *et al*. 2000, Bonneaud *et al*. 2003). These studies demonstrate a trade-off between immunity and behaviours that contribute directly to reproductive success. In addition, it should also be noted that immunocompetence can influence

fecundity directly. Lines of domestic chickens bred for high antibody production have lower egg production and overall reduced lifetime fitness (Siegel *et al*. 1982, Martin *et al*. 1990).

b) Sex-biased Immunocompetence

Across a wide range of animals, males tend to have higher rates of parasitism than females (Poulin 1996, Zuk and McKean 1996, Moore and Wilson 2002), and these observations were key to recognizing sex differences in immunity. Indeed, there appears to be a similar pattern for immune function: males tend to have poor immune function compared with females in many groups of animals (Zuk and McKean 1996, Klein 2000, Nunn *et al*. 2009) and this pattern could be explained by the strength and direction of sexual selection and reproductive investment.

Bateman's Principle was borne from studies of insects (Bateman 1948), where parental investment is largely non-existent and anisogamy is sufficient to drive fundamental sex differences in reproductive investment. However, the presence of parental care in females (i.e., incubation/chick rearing in birds) increases the relative investment by females in 'current' reproduction which could diminish the perceived differences between the sexes in this regard; with high levels of female-biased parental care there may in fact be little difference between the sexes in the amount of annual investment. Nevertheless, the premise behind Bateman's Principle is likely still valid in species exhibiting female-biased parental care because those species do still exhibit sex-biased immunocompetence (e.g., Møller *et al*. 1998: species listed in Appendix A; as well as in mammals where parental care is largely female-biased: Nunn *et al*. 2009).

c) Comparative Studies

Comparative studies of POL provide some of the best evidence of reproduction-immune system trade-offs because they demonstrate a relationship between life history traits and immune

function at an evolutionary scale. Fast-paced species undergo more intense bouts of reproduction (i.e., produce larger clutches, increased competition for mates) and have faster developmental rates, both of which require resources that could be used for immune function and/or inhibit the development of the immune system (Sheldon and Verhulst 1996, Lee 2006). Using cell-mediated immunity, Tella *et al*. (2002) demonstrated that species with greater immune function had longer incubation and nestling periods, longer lifespan, slower growth rate and lower adult mortality. Although the results are compelling across a very broad group of species, the authors failed to use phylogenetically informed analyses. Given the strength of some of the phylogenetic relationships, these particular results are perhaps questionable. Nevertheless, other studies confirm that traits related to POL correlate with immunity in the predicted manner: basal metabolic rate is negatively related to bactericidal activity (Tieleman *et al*. 2005); species with longer incubation periods have higher cell-mediated immune response (Palacios and Martin 2006) and more natural antibodies (Lee *et al*. 2008). To date though, these comparative studies have focussed largely on passerine birds.

Ecoimmunological trade-offs are complicated by the complexity of the immune system and its various functions. Down-regulation of one aspect of the immune system may be countered by up-regulation or maintenance of another (Roberts and Peters 2009, Kuhlman and Martin 2010). Trade-offs among aspects of immunity should be the case if there are notable differences in the costs associated with maintaining specific arms of the immune system. For example, aggressive male-male interactions may result in injuries best served by a strong first-line defence (i.e., innate immunity) as opposed to acquired immune responses (Zuk and Johnsen 1998). Similarly,

faster-paced species may invest more in first line defences while slower-paced species invest in more complex immune responses (Lee 2006, Lee *et al*. 2008).

Hormones, Immune Function and the Immunocompetence Handicap

Immune investment and expression are regulated via a number of physiological pathways that involve mediators such as cytokines and hormones. Arguably, many hormones have been demonstrated, or at least proposed, to have the ability to modulate the immune system (O'Neal 2013). However, two in particular, testosterone and corticosterone, have received the most attention in ecology and evolution for their potential role in immunosuppression (Folstad and Karter 1992, Møller 1995, Roberts *et al*. 2007).

One of the most influential concepts in evolutionary biology in the past two decades is the Immunocompetence Handicap Hypothesis (ICHH; Folstad and Karter 1992). Folstad and Karter (1992) argued that there is a hormonal link that unites the Hamilton-Zuk hypothesis (Hamilton and Zuk 1982) with the notion of a Zahavian handicap (Zahavi 1975). Specifically, Zahavi (1975) posited that in order for traits to be honest signals they must impose a handicap such that only the best individuals can overcome the disadvantages of possessing the trait. Hamilton and Zuk (1982) made the seminal observation that plumage colouration is positively related to parasite load. Folstad and Karter (1992) argued that testosterone enforces honest signals because it has a role in the expression of secondary sexual traits, such as plumage colouration, and is purportedly immunosuppressive, the handicap.

Fundamentally, the ICHH was founded based on four observations: i) that males tend to have higher parasite loads and poorer immunocompetence than females, ii) that males have higher testosterone levels than females, iii) that males possess elaborate sexual signals, the expression of which is mediated by testosterone, and iv) that testosterone is immunosuppressive.

While being pervasive and well entrained in the evolutionary ecology literature, several tenets of the ICHH remain contentious. Most notably, a meta-analysis did not find broad support for an immunosuppressive effect of testosterone (Roberts *et al*. 2004), and many experimental studies have yielded equivocal results (e.g., Evans *et al*. 2000, Roberts *et al*. 2007, Roberts *et al*. 2009). Other authors point out that sex-biased parasitism and immunocompetence occurs in invertebrates, where testosterone is not present, suggesting that the mechanism need not rely on a testosterone-mediated handicap alone (e.g., Nunn *et al*. 2009). Other points of contention include that testosterone is not responsible for producing sexually dimorphic plumage in several commonly studied groups of birds, including passerines (Kimball and Ligon 1999), and that there is a temporal disconnect between elevated testosterone during the breeding season, and when moult occurs. Finally, others argue that the ICHH is untestable because it makes no clear predictions (Braude *et al*. 1999, Oliveira 2004). Despite these points, the ICHH remains popular, having garnered well over 60 citations per year over the last ten years.

More recently, other hormones have been implicated in the ICHH mechanism as well (e.g., leptin; Alonzo-Alvarez *et al*. 2007), chief among them corticosterone (Møller 1995, Roberts *et al*. 2007). Corticosterone primarily functions to liberate energy stores and initiate behaviours that promote survival during stressful events (Wingfield *et al*. 1990, Remage-Healey and Romero 2001), and immunosuppression is a common consequence of elevated corticosterone (Råberg *et al*. 1998, Buchanan 2000, Sapolsky *et al*. 2000). Both hormones are elevated during the breeding season (e.g., Breuner and Orchinik 2001) and experimentally administering testosterone has been shown to elicit a simultaneous increase in corticosterone (e.g., Ashley *et al*. 2009). Thus, the actions of testosterone and corticosterone may be difficult to

differentiate, and testosterone may actually play an indirect role in mediating an ICHH-like mechanism (Owen-Ashley *et al*. 2004).

The most fundamental aspect of the ICHH is that it is a handicap that maintains the honest expression of secondary sexual characters by impairing the immune system (Folstad and Karter 1992). Handicaps are not the costs associated with trait production, but rather are additional costs without which the trait would not be reliably expressed (Grafen 1990, Maynard Smith 1991, Folstad and Karter 1992, Zahavi and Zahavi 1997, Számadó 2011). The notion that the ICHH is required to maintain signal honesty forms the basis of how I approach this dissertation.

At the time of writing this dissertation, the ICHH had been cited well over 1300 times. A search of those citations revealed the term "ecological immunology" fewer than 70 times. Thus, rarely is the ICHH discussed in light of ecological-based trade-offs involving the immune system. However, several notable studies have drawn clear divisions between Bateman-like mechanisms and handicaps (Rolff 2002, Nunn *et al*. 2009, Kelly and Alonzo 2010), or between the inherent reliability of signals and handicaps (Hill 2011, Számadó 2011, Emlen *et al*. 2012), highlighting that these mechanisms should indeed be thought of as being distinctly separate. Thus, I secondarily approach the dissertation by contrasting handicaps with ecological mechanisms explaining immune investment.

Dissertation Outline

Hamilton and Zuk (1982) demonstrated that species with brighter plumage have more parasites. Do they have more parasites because they are less immunocompetent, or do they have greater investment in immunocompetence because they face greater parasite pressure? Alternatively, do species that invest more in reproduction, particularly in the form of 'costly' secondary sexual

traits, suffer an immunological cost for doing so? To my knowledge, no studies have explicitly followed up on Hamilton and Zuk, which used variation in parasite burden to demonstrate the underlying relationship between immunocompetence and plumage colour.

In Chapter 2, my primary interest was in whether species possessing more elaborate secondary sexual traits suffer an immunological cost for doing so, as predicted by the ICHH (Folstad and Karter 1992). Duller species of birds do appear to live longer than brighter species (Promislow *et al*. 1992, Promislow *et al*. 1994), but this is contrary to the observation that within species, females prefer to mate with brighter (Andersson 1994), more immunocompetent males (e.g., Møller *et al*. 1999). Given our current knowledge of immune investment there is a paradox of sorts between the expectations for investment in longevity, investment in reproduction via secondary sexual traits, and mate choice for superior ornaments. Thus, the goal of Chapter 2 was to reconcile the relationships among immune investment, longevity and the expression of secondary sexual traits using a comparative approach. I was secondarily interested in explaining immune investment in terms of POL, because POL is directly related to longevity. Furthermore, using secondary sexual traits (i.e., plumage colour) as a proxy for investment in current reproduction, species with 'dull' plumage should also demonstrate characteristics of slower POL, and higher immune investment; 'brighter' species should demonstrate characteristics of faster POL and have lower levels of immune investment.

In Chapter 2, I investigated this issue using a comparative framework in parrots (Psittaciformes). Parrots are a particularly good model to use because they are a relatively speciose order for which longevity is reasonably well documented. Because they are commonly kept in captivity, immunocompetence can be measured in a relatively standardized manner across species, in relatively benign environments (limiting the influence of current infection on

immune measures), such that evolutionarily derived levels of immune investment can be measured. Furthermore, parrots are good models because they have surprisingly invariable life histories – most are socially monogamous, are constrained to the tropics, most consume fruit and seeds, and all nest in cavities of one form or another – all traits that could influence immune investment. Cavity nesting also liberates the need for camouflage in females (most species of parrots are sexually monochromatic), which should relax the expression of male plumage due to sexual conflict, theoretically allowing their signal expression to more accurately reflect the strength of sexual selection via mate choice.

Above I briefly reviewed the possible role for corticosterone in determining sex differences in immunity and in the ICHH mechanism. Interestingly, sex differences in the adrenocortical stress response have been linked to incubation duties among shorebirds (O'Reilly and Wingfield 2001). Attenuation of the stress axis could ensure resilience to environmental stressors. Among incubating birds for example, corticosterone could influence abandonment rates, which could ultimately explain why the incubating sex tends to have lower stress induced levels of corticosterone (O'Reilly and Wingfield 2001). As such, sex differences in corticosterone, which can be immunosuppressive in both acute and chronic exposure to stress in wild birds (Buchanan 2000, Sapolsky *et al*. 2000, Matson *et al*. 2006, Buehler *et al*. 2008, Merrill *et al*. 2012, Chin *et al*. 2013), could be explained either by courtship activities, incubation activities, or both. In Chapter 3, I investigated relationships between corticosterone levels and sex, as well as corticosterone levels in relation to nest defence behaviours and hatch success in two species of shorebirds. Given the potential immunosuppressive role of corticosterone, understanding these

mechanisms will contribute to our understanding of how sex specific life histories, and sex-biased immunocompetence, are mediated by hormones.

Bateman's Principle suggests that males maximize fitness by investing in high rates of current reproduction, whereas females maximize fitness by future reproduction (Bateman 1948; see also Tang-Martínez 2013) and as a consequence, males are expected to trade-off reproduction and immunity such that males are immunocompromised relative to females (Rolff 2002). Whereas male-biased immunosuppression generally appears to be the case across a broad range of taxa (Zuk and McKean 1996, Klein 2000, Nunn *et al*. 2009), in vertebrates, immunosuppression in males has also been explained as a result of the actions of testosterone via the ICHH (Folstad and Karter 1992, Rolff 2002). However, because in vertebrates males possess high levels of testosterone, have sexual signals that are mediated by testosterone and are immunosuppressed, there is a challenge to distinguish between a testosterone mediated handicap and a Bateman's-like process. A critical 'test' of the causes of sex differences in immunity, as they relate to Bateman's Principle or a testosterone-mediated handicap, is to remove 'maleness' from the equation. The means to achieve this is via a natural experiment of sorts: to research a species in which sex roles are reversed. In Chapter 4, I study Red phalaropes (*Phalaropus fulicarius*) to determine the direction of sex-biased immunocompetence and the role of testosterone and corticosterone in explaining immune function. Female phalaropes possess all the hallmarks that are typically attributed to high investment in current reproduction in males: they are larger than males, are more brightly coloured and have higher reproductive potential. Because of this, I expect females to exhibit lower indices of immune function than males despite high levels of

testosterone in males. This would demonstrate a predicted uncoupling between testosterone and its immunosuppressive handicap.

In Chapter 5, I then tested for condition-dependence of plumage colouration in male and female phalaropes. Sexual signals, such as plumage colour, should carry information about the quality of the signaller; signals that do not convey accurate information should not be used in mate choice. Whereas much research has been done on testosterone-mediated traits and 'honest' expression in males, fewer studies have considered the expression of both sexes simultaneously and, to my knowledge, no studies have yet looked at plumage expression in a sex-role reversed species. This chapter follows from Chapter 4 in that it asks how immune measures, and condition in general, relate to plumage expression in a species where males have higher levels of testosterone than females, yet females are more colourful. Finally in Chapter 6, I investigate mate choice in phalaropes to determine whether plumage and other traits are used in mate selection.

In summary, this dissertation broadly covers issues relating to sexual selection, sexual signalling and life history theory, whereby I specifically investigated trade-offs involving reproduction and immune function. To achieve these aims, I examined relationships among plumage expression, life history and immunity across species (Chapter 2), between sexes (Chapter 4) and among individuals within a sex (Chapter 5). Furthermore, I considered the role of hormones in explaining sex differences in immune function (Chapter 4) and plumage expression (Chapter 5), as well as the role of explaining important life history differences between the sexes (Chapter 3). Finally, I looked at the pattern of mate choice based on plumage characters and body size (Chapter 6).

Literature Cited

Abad-Gomez, J.M., J.S. Gutierrez, A. Villegas, J.M. Sanchez-Guzman, J.G. Navedo and J.A. Masero. 2013. Time course and metabolic costs of a humoral immune response in the Little Ringed Plover *Charadrius dubius*. Physiol Biochem Zool 86: 354-360.

Alonzo-Alvarez, C., S. Bertrand, and G. Sorci. 2007. Energetic reserves, leptin and testosterone: a refinement of the immunocompetence handicap hypothesis. Biol Lett 3: 271-274.

Andersson, M. 1994. Sexual Selection. Princeton: Princeton University Press. 624p.

Ashley, N.T., Q.R. Hays, G.E. Bentley and J.C. Wingfield. 2009. Testosterone treatment diminishes sickness behavior in male songbirds. Horm Behav 56: 169-176.

Bateman, A.J. 1948. Intra-sexual selection in *Drosophila*. Heredity 2: 349-368.

Benito, M.M. and J. Gonzalez-Solis. 2007. Sex ratio, sex-specific chick mortality and sexual size dimorphism in birds. J Evol Biol 20: 1522–1530.

Bonneaud, C., J. Mazuc, G. Gonzalez, C. Haussy, O. Chastel, B. Faivre and G. Sorci. 2003. Assessing the cost of mounting an immune response. Am Nat 161: 367-379.

Bonneaud, C., S.L. Balenger, G.E. Hill and A.F. Russell. 2012. Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. Mol Ecol 21: 4787-4796.

Boonstra, R., C.J. McColl and T.J. Karels. 2001. Reproduction at all costs: The adaptive stress response of male arctic ground squirrels. Ecology 82: 1930-1946.

Braude, S., Z. Tang-Martinez and G.T. Taylor. 1999. Stress, testosterone, and the immunoredistribution hypothesis. Behav Ecol 10: 345-350.

Breuner, C.W. and M. Orchinik. 2001. Seasonal regulation of membrane and intracellular receptors in the house sparrow brain. J Neuroendocrinol 13: 412-420.

Brzek, P. and M. Konarzewski. 2007. Relationship between avian growth rate and immune response depends on food availability. J Exp Biol 210: 2361-2367.

Buchanan, K.L. 2000. Stress and the evolution of condition-dependent signals. Trends Ecol Evol 15: 157-160.

Buehler, D.M., N. Bhola, D. Barjaktarov. W. Goymann, I. Schwabl, B.I. Tieleman and T. Piersma. 2008. Constitutive immune function responds more slowly to handling stress than corticosterone in a shorebird. Physiol Biochem Zool 81: 673-681.

Burness, G., C. Armstrong, T. Fee and E. Tilman-Schindel. 2010. Is there an energetic-based trade-off between thermoregulation and the acute phase response in zebra finches? J Exp Biol 213: 1386-1394.

Chin, E.H., J.S. Quinn and G. Burness. 2013. Acute stress during ontogeny suppresses innate, but not acquired immunity in a semi-precocial bird (*Larus delawarensis*). Gen Comp Endocrinol 193: 185-192.

Emlen, D.J., I.A. Warren, A. Johns, I. Dworkin and L.C. Lavine. 2012. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. Science 337: 860-864.

Evans, M.R., A.R. Goldsmith and S.R.A. Norris. 2000. The effects of testosterone on antibodyproduction and plumage colouration in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 47: 155-163.

Folstad, I. and A.J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603-622.

Grafen, A. 1990. Biological signals as handicaps. J Theor Biol 144: 517-546.

Gutierrez, J.S., J.A. Masero, J.M. Abad-Gomez, A. Villegas and J.M. Sanchez-Guzman. 2011. Metabolic consequences of overlapping food restriction and cell-mediated immune response in a long-distance migratory shorebird, the little ringer plover *Charadrius dubius*. J Avian Biol 42: 259-265.

Hamilton, W.D. and M. Zuk. 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Hanssen, S.A. 2006. Costs of an immune challenge and terminal investment in a long-lived bird. Ecology 87: 2440-2446.

Harshman, L.G. and A.J. Zera. 2007. The cost of reproduction: the devil in the details. Trends Ecol Evol 22: 80-86.

Hasselquist, D. and J.-Å. Nilsson. 2012. Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? Anim Behav 83: 1303-1312.

Hill, G.E. 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. Ecol Lett 14: 625-634.

Kelly, N.B., and S.H. Alonzo. 2010. Does a trade-off between current reproductive success and survival affect the honesty of male signalling in species with male parental care? J Evol Biol 23: 2461-2473.

Kimball, R.T. and J.D. Ligon. 1999. Evolution of avian plumage dichromatism from a proximate perspective. Am Nat 154: 182-193.

King, M.O. and D.L. Swanson. 2013. Activation of the immune system incurs energetic costs but has no effect on the thermogenic performance of house sparrows during acute cold challenge. J Exp Biol 216: 2097-2102.

Klasing, K.C. 2004. The costs of immunity. Acta Zoologica Sinica 50: 961-969.

Klein, S.L. 2000. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci Biobehav Rev 24: 627-638.

Kluger, M.J., W. Kozak, C.A. Conn, L.R. Leon and D. Soszynski. 1998. Role of fever in disease. Ann N Y Acad Sci 29: 224-233.

Knowles, S.C.L, S. Nakagawa and B.C. Sheldon. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. Funct Ecol 23: 405-415.

Kuhlman, J.R. and L.B. Martin. 2010. Captivity affects immune redistribution to skin in a wild bird. Funct Ecol 24: 830-837.

Lee, K.A. 2006. Linking immune defenses and life history at the levels of the individual and the species. Integr Comp Biol 46: 1000-1015.

Lee, K.A., M. Wikelski, W.D. Robinson, T.R. Robinson and K.C. Klasing. 2008. Constitutive immune defences correlate with life-history variables in tropical birds. J Anim Ecol 77: 356-363.

Liker, A. and T. Székely. 2005. Mortality costs of sexual selection and parental care in natural populations of birds. Evolution 59: 890–897.

Liker, A., R.P. Freckleton and T. Székely. 2013. The evolution of sex roles in birds is related to adult sex ratio. Nat Commun 4: 1587.

Martin, A., E.A. Dunnington, W.B. Gross, W.E. Briles, R.W. Briles and Siegel, P.B. 1990. Production traits and alloantigen systems in lines of chickens selected for high or low antibody-responses to sheep erythrocytes. Poult Sci 69: 871–878.

Martin, L.B., A. Scheuerlein and M. Wikelski. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc Biol Sci 270: 153-158.

Martin, L.B., D.M. Hawley and D.R. Ardia. 2011. An introduction to ecological immunology. Funct Ecol 25: 1-4.

Matson, K.D., B.I. Tieleman and K.C. Klasing. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. Physiol Biochem Zool 79: 556-564.

Maynard Smith, J. 1991. Honest signalling: the Philip Sidney Game. Anim Behav 42: 1034-1035.

Merrill, L., F. Angelier, A.L. O'Loghlen, S.I. Rothstein and J.C. Wingfield. 2012. Sex-specific variation in brown-headed cowbird immunity following acute stress: a mechanistic approach. Oecologia 170: 25-38.

Møller, A.P. 1995. Hormones, handicaps and bright birds. Trends Ecol Evol 10: 121.

Møller, A.P., G. Sorci and J. Erritzøe. 1998. Sexual dimorphism in immune defense. Am Nat 152: 605-619.

Møller, A.P., P. Christe and E. Lux. 1999. Parasitism, host immune function and sexual selection. Q Rev Biol 74: 3-20.

Moore, S.L. and K. Wilson. 2002. Parasites as a viability cost of sexual selection in natural populations of mammals. Science 297: 2015-2018.

Nunn, C.L., P. Lindenfors, E.R. Pursall and J. Rolff. 2009. On sexual dimorphism in immune function. Phil Trans R Soc B 364: 61-69.

Oliveira, R.F. 2004. Social modulation of androgens in vertebrates: mechanisms and function. Adv Study Behav 34: 165-239.

O'Neal, D.M. 2013. Eco-endo-immunology across avian life history stages. Gen Comp Endocrinol 190: 105-111.

O'Reilly, K.M. and J.C. Wingfield, 2001. Ecological factors underlying the adrenocortical response to capture stress in arctic-breeding shorebirds. Gen Comp Endocrinol 124: 1-11.

Owen-Ashley, N.T., D. Hasselquist and J.C. Wingfield. 2004. Androgens and the immunocompetence handicap hypothesis: Unraveling direct and indirect pathways of immunosuppression in Song Sparrows. Am Nat 164: 490-505.

Palacios, M.G. and T.E. Martin. 2006. Incubation period and immune function: a comparative field study among coexisting birds. Oecologia 146: 505-512.

Poulin, R. 1996. Sexual inequalities in helminth infections: a cost of being male? Am Nat 147: 287-295.

Promislow, D.E.L., R. Montgomerie and T.E. Martin. 1992. Mortality costs of sexual dimorphism in birds. Proc Biol Sci 250: 143-150.

Promislow, D.E.L., R. Montgomerie and T.E. Martin. 1994. Sexual selection and survival in North American waterfowl. Evolution 48: 2045-2050.

Råberg, L., M. Grahn, D. Hasselquist and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. Proc Biol Sci 265: 1637–1641.

Råberg, L., J. Å. Nilsson, P. Ilmonen, M. Sternman and D. Hasselquist. 2000. The cost of an immune response: vaccination reduces parental effort. Ecol Lett 3: 382-386.

Remage-Healey, L. and L.M. Romero. 2001. Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. Am J Physiol Regul Integr Comp Physiol 281: R994– R1003.

Reznick, D.N. 2010. *The Origin Then and Now. An Intepretative Guide to the Origin of Species.* Princeton: Princeton University Press.

Roberts, M.L. and A. Peters. 2009. Is testosterone immunosuppressive in a condition-dependent manner? An experimental test in blue tits. J Exp Biol 212: 1811-1818.

Roberts, M.L., K.L. Buchanan and M.R. Evans. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim Behav 68: 227-239.

Roberts, M.L., K.L. Buchanan, D. Hasselquist and M.R. Evans. 2007. Effects of testosterone and corticosterone on immunocompetence in the zebra finch. Horm Behav 51: 126-134.

Roberts, M.L., K.L. Buchanan, M.R. Evans, R.H. Marin and D.G. Satterlee. 2009. The effects of testosterone on immune function in quail selected for divergent plasma corticosterone response. J Exp Biol 212: 3125-3131.

Roff, D.A. 1992. *The Evolution of Life Histories: Theory and Analysis* . New York, Chapman & Hall.

Rolff, J. 2002. Bateman's principle and immunity. Proc Biol Sci 269: 867-872.

Rose, M.R. and T.J. Bradley. 1998. Evolutionary physiology of the cost of reproduction. Oikos 83: 443-451.

Sapolsky, R.M., M. Romero and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparatory actions. Endocr Rev 21: 55-89.

Schulenburg, H., J. Kurtz, Y. Moret and M.T. Siva-Jothy. 2009. Introduction. Ecological immunology. Phil Trans R Soc B 364: 3-14.

Sheldon, B.C. and S. Verhulst. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 11: 317–321.

Siegel, P.B., W.B. Gross and J.A. Cherry. 1982. Correlated responses of chickens to selection for production of antibodies to sheep erythrocytes. Anim Blood Groups Biochem Genet 13: 291–297.

Stearns, S.C. 1992. *The Evolution of Life Histories*. Oxford, Oxford University Press.

Számadó, S. 2011. The cost of honesty and the fallacy of the handicap principle. Anim Behav 81: 310.

Tang-Martínez, Z. 2012. Repetition of Bateman challenges the paradigm. Proc Natl Acad Sci U.S.A. 109: 11476-11477.

Tella, J.L., A. Scheuerlein and R.E. Ricklefs. 2002. Is cell-mediated immunity related to the evolution of life-history strategies in birds? Proc Biol Sci 269: 1059-1066.

Tieleman, B.I., J.B. Williams, R.E. Ricklefs and K.C. Klasing. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. Proc Biol Sci 272: 1715-1720.

Wingfield, J.C., H. Schwabl and P.W. Mattocks Jr. 1990. Endocrine mechanisms of migration. In: Gwinner, E. (Ed.), Bird Migration: Physiology and Ecophysiology. Springer-Verlag, New York.

Zahavi, A. 1975. Mate selection: a selection for a handicap. J Theor Biol 53: 205-214.

Zahavi, A., and Zahavi, A. 1997. The Handicap Principle. New York: Oxford University Press.

Zuk, M. and K.A. McKean. 1996. Sex differences in parasite infections: Patterns and processes. Int J Parasitol 26: 1009-1024.

Zuk, M. and T.S. Johnsen. 1998. Seasonal changes in the relationship between ornamentation and immune response in jungle fowl. Proc Biol Sci 265: 1631-1635.

Zuk, M. and A.M. Stoehr. 2002. Immune defence and host life history. Am Nat 160: S9-S22.

CHAPTER 2: Immune investment is explained by sexual selection and pace-of-life, but not longevity in parrots (Psittaciformes).

Abstract

Investment in current reproduction should come at the expense of traits promoting future reproduction, such as immunity and longevity. To date, comparative studies of pace-of-life traits have provided some support for this, with slower paced species having greater immune function. Another means of investment in current reproduction is through secondary sexual characters (SSC). Investment in SSC's is considered costly, both in terms of immunity and longevity, with greater costs being borne by species with more elaborate traits. Yet within species, females prefer more ornate males and those males are typically immunologically superior. Because of this, predictions about the relationship between immunity and SSC's across species are not clear. If traits are costly, brighter species should have reduced immune function, but the opposite is true if SSC's arise from selection for more immunocompetent individuals. My approach was to investigate immune investment in relation to SSC's, pace-of-life and longevity while considering potentially confounding ecological factors. To do so I assessed leukocyte counts from in a novel group, the Psittaciformes. Investment in SSC's best explained investment in immunity: species with brighter plumage had higher leukocyte counts and those with a greater degree of sexual dichromatism had fewer. Ecological variables and pace-of-life models tended to be poor predictors of immune investment. However, shorter incubation periods were associated with lower leukocyte counts supporting the notion that species with a fast pace-of-life invest less in immunity. These results suggest that investment in reproduction in terms of fast pace-of-life and sexual dichromatism results in reduced immunity, however, investment in plumage colour per se does not impose a cost on immunity across species.

Introduction

The immune system has evolved in response to energetic trade-offs with other life history traits and with the risk of infection. The balance between maintaining optimal immune defence and aspects of life history, such as reproduction, has generated considerable research in the past couple of decades (e.g., Lochmiller and Deerenberg 2000, Norris and Evans 2000).

The fundamental prediction of life history theory is that individuals trade-off among activities that compete for resources, such as reproduction and survival (Williams 1966, Stearns 1992). Since immune function is an important aspect of self-maintenance that is energetically costly (Råberg *et al*. 2000, Martin *et al*. 2003), investment in immunity should be traded against investment in reproduction (Sheldon and Verhulst 1996, Zuk and Stoehr 2002). For many species, emphasis on reproduction produces exaggerated secondary sexual characters (SSC) that compete for resources that would otherwise be used for self-maintenance. For example, adult mortality appears to be related to costs associated with bearing SSC's, such as large body size in mammals (Promislow 1992, Moore and Wilson 2002) or bright plumage in birds (Promislow *et al*. 1992, Promislow *et al*. 1994). In the case of birds, costs associated with bearing bright plumage are considered a fundamental aspect of signalling theory (Zahavi 1975), and the notion that elaborate signal traits impose costs directly on the immune system is well established (Zuk and Stoehr 2002, Folstad and Karter 1992).

If investment in SSC's is costly, we might expect these traits to be negatively related to immune investment and longevity across species. Yet within avian species, males with more elaborate plumage are preferred by females (Andersson 1994) and are often immunologically superior to, and have fewer parasites than, duller individuals (e.g., Møller *et al*. 1999). Across species, those with more parasites tend to have brighter plumage (Hamilton and Zuk 1982), possibly resulting from stronger selection for more immuno-competent individuals in species

experiencing higher infection rates (although the reverse argument can also be employed: costly plumage traits could result in reduced immune investment across species resulting in high rates of parasitism). As a result, brighter species could have greater immune investment than duller ones, and consequently greater longevity, which is contrary to the notion of costly investment in SSC's.

The main goal of this study is to address a paradox of sorts: do species investing in elaborate plumage traits face costs for doing so and hence have reduced immunity, or does plumage exaggeration signal greater immune ability against increased infection threats? I measured sexual selection in terms of overall plumage exaggeration, as well as the degree of sexual size dimorphism and dichromatism. I predict that species with more elaborate plumage will have greater immune investment, in keeping with the idea that greater intra-sexual selection for good quality individuals results in more elaborate plumage at the species level. Alternatively, I predict that more pronounced sexual dimorphisms in body size and plumage result in reduced immune investment as a consequence of conflicting sexual strategies. For example, in the studies cited above, costs reported for SSC's were measured as sexual size dimorphism (Promislow 1992, Moore and Wilson 2002) and sexual dichromatism (Promislow *et al*. 1992), rather than overall expression (but see Promislow *et al*. 1994). Sexual dimorphisms in appearance are likely to evidence sexual conflict for other traits resulting in compromises to investment in self-maintenance at the species level (Arnqvist and Rowe 2005).

A secondary goal of this paper is to place sexual selection and longevity in the context of other life history traits more typically studied in the recent comparative eco-immunology literature. Longevity is rarely used in these studies, and instead immune investment is often investigated in the context of traits associated with pace-of-life. Pace-of-life is directly related to

longevity: a slow pace-of-life is typified by species with long developmental periods and late maturation, low reproductive potential and long life span (Promislow and Harvey 1990). On the fast end of the life history continuum, species are expected to invest more in reproduction, such as increased clutch size and possibly SSC's (Bonduriansky *et al*. 2008), and less in immunity. However, to date the results from pace-of-life studies have been somewhat disparate with slower pace-of-life showing both superior and inferior immune function depending on the measure of immunity used (Tella *et al*. 2002, Tieleman *et al*. 2005, Palacios and Martin 2006, Lee *et al*. 2008).

The relationship between plumage colour and parasites demonstrated by Hamilton and Zuk (1982) was initially criticized because it lacked potentially confounding ecological variables that could influence either plumage colour or infection risk (Read 1987). Because of this I included four ecological variables – group size, habitat, diet, region – that could help explain patterns in plumage colour, infection risk and longevity.

Although the interplay between infections and immunity is complex, the immune system is expected to evolve in concert with the risk of infection. Transmission rates of infections increase with group size (Côté and Poulin 1995), and as a consequence so should investment in immunity. In support of this, immune investment is higher in species forming larger social groups (birds: Møller *et al*. 2001, mammals: Nunn 2002, Nunn *et al*. 2003) and with increased mating promiscuity (mammals: Nunn 2002, Nunn *et al*. 2003). However, infection rates could also be indirectly related to plumage colour if signal intensity increases with social group size as a means of communication among individuals in large flocks.

For habitat, diet and region specific expectations are not as clear: *Habitat*) The perception of colour depends upon the nature of the light environment, and the light environment differs

with light penetration in different habitats (Endler 1993). In order to maximize signal efficiency, selection on plumage colour differs with habitat use (McNaught and Owens 2002), where habitat use is defined in the context of light penetration. *Diet*) Dietary antioxidants, such as from fruit, can play an important role in bolstering immunity (Catoni *et al*. 2008). Diet can explain patterns in longevity as well, with the degree of frugivory accounting for both shorter (Peach *et al*. 2001) and longer (Munshi-South and Wilkinson 2006) lifespan among different groups of birds. *Region*) Immune investment varies with latitude (Møller 1998), between tropical and non-tropical species (Møller and Erritzøe 1998), and between island and continental species (Matson 2006), and parasite infection rates differ between continental regions (Valkiunas 2004). I included species' native range in order to account for geographic differences in infection risk and immune investment.

The immune system is a complex trait and as such cannot be fully described by a single measure (Lee 2006). To date, few large comparative studies have used multiple immune measures, as it is logistically difficult to study at a broad phylogenetic and geographic scale. In this study, I assessed immune investment using leukocyte counts collected from captive individuals. Leukocytes are comprised of five cell types that function in both the innate and acquired immune responses (Roitt *et al*. 1993). Leukocytes have not been used in avian comparative studies, but are frequently used in mammalian studies where leukocyte counts are often explained by variables relating to disease risk, sexual selection and life history traits (Nunn *et al*. 2001, Nunn 2002, Semple *et al* 2002, Nunn *et al*. 2003, Anderson *et al*. 2004, Nunn *et al*. 2009).

I conducted this study on parrots (Psittaciformes). So far, passerines have been the focus of pace-of-life studies, and parrots are not often studied in the context of sexual selection though

they are ideal for a number of reasons. Parrots are long-lived and have elaborate plumage characters. Because they are readily kept in captivity, particularly in zoos, comparative immune measures are available for a large number of species, and captive lifespan has been reported for many species (Brouwer *et al*. 2000). The Psittaciformes are a relatively speciose group (approximately 375 species) but life history traits, such as diet and nesting location, are relatively invariable across species compared to passerines (del Hoyo *et al*. 1997, Juniper and Parr 1998). That virtually all are cavity nesters of some sort means there is relaxed selection on female crypsis meaning that elaboration of plumage traits may more accurately reflect the strength of selection for those traits. Although there is little information available about the frequency of promiscuity in wild parrots, they are virtually all socially monogamous, forming and maintaining pair bonds during the breeding season (del Hoyo *et al*. 1997). As a result, hidden sources of sexual selection that may not explicitly be manifest as selection on plumage colouration are limited across species.

Methods

Leukocyte Data

I collected information for a total of 66 parrot species from 36 genera for which leukocyte data were available. I used baseline leukocyte values published in the International Species Information System (ISIS; Reference Ranges for Physiological Values in Captive Wildlife, 2002, Apple Valley, Minnesota Zoological Garden, MN, USA) as a measure of immunity. ISIS is a dataset of baseline haematological reference values taken from healthy captive animals from zoos and other housing institutions. Species mean values represent averages of data pooled from different member institutions (i.e. locations), and for both males and females. To investigate quality of the dataset I used coefficients of variation (CV) of total leukocyte counts. CV's were less than 1 (0.33-0.75) for all cases except for one (1.22) and so were taken to represent

consistent counts across individuals and member institutions. Inclusion of the single species with high CV did not affect the results and so it was included in the analysis.

Plasma leukocyte counts increase for various reasons including in response to immunological challenge. However, ISIS data are collected only from generally healthy individuals to be used as part of a Complete Blood Count to assess sickness when monitoring captive animals. As such, counts from captive animals are likely less variable and may better reflect evolutionarily derived investment than measures from wild specimens.

One species was excluded from my analysis because it represented an extremely unique life history. *Nestor notabilis* is flightless, polygynous, and known to consume carrion. Flightlessness likely liberates energy for other physiological process such as immunity, and because it is ground-dwelling it is also likely constrained in the expression of bright plumage, which in this species is dull, despite facing high levels of sexual selection. Furthermore, increased disease transmission from scavenging carcasses may result in selection on immunity not related to sexual selection.

Life-history and Behavioural Traits

Lifespan estimates (n=56 for which leukocyte data were available) were taken from Brouwer *et al*. (2000). These estimates are the longest reported lifespans (years) for captive parrots, which can be interpreted as maximum potential lifespan (MLSP) for the species. Birds typically remain reproductively active until death (Holmes and Austad 1995), and intrinsic mortality in captivity reflects rates of intrinsic mortality in the wild (Ricklefs 2000). For these reasons, traits associated with investment in longevity, such as immunity, are likely to relate to MLSP.

Life history traits were gathered through literature searches: body mass (g) was averaged over several sources (del Hoyo *et al*. 1997, Juniper and Parr 1998, as well as from ISIS itself),

sexual size dimorphism (Forshaw 1973) and social group size (Juniper and Parr 1998, Forshaw 1973) was categorically defined as: small (2-14 birds), medium (15-50 birds), medium-large (50-200) and large (>200). All other life history traits (see Table 1) were averaged across sources where possible (del Hoyo *et al*. 1997, Juniper and Parr 1998, Dunning 2008) except for a small number of species where additional sources were needed (Low 1998, Alderton 2000). Sexual size dimorphism and dichromatism were categorized as absent, minimal or present. Diet was defined as: granivorous, frugivorous, generalist/omnivore and nectar feeding based on the majority composition of the diet. Habitat was categorized as either open, semi-open or closed and geographic region was based on the majority of the species natural range (grouped as America, Africa, Asia, Indonesia/Papua New Guinea/Malaysia and Australia). Life history traits are reported for wild individuals except for body mass.

Colouration

Several elements of plumage colouration and exaggeration of physical traits were ranked by five independent observers from painted colour plates (Forshaw 1973). Each species was scored from 1-5 (low to high) based on: i) Brightness - overall brightness of the plumage; ii) Contrast - the overall impression of the degree of contrasting plumage traits, iii) Complexity - the complexity of appearance including the number of colours and complexity of colour patterns including non-feather colour traits such as pigmented skin and feather adornments such as crests; iv) Non-background - the relative amount of non-background colouration could be suggestive of the strength of selection on traits that may not be otherwise bright or complex; v) The amount of Red/Orange/Yellow used in plumage traits. Where species were sexually dichromatic, observers were instructed to assess only male plumage colouration.

Phylogeny

Phylogenetic hypotheses for parrots are relatively well established for higher taxonomic levels however species specific trees are either not available for a broad range of species or suffer discordances among studies. I used the phylogeny outlined by Mayr (2010) which is derived from Wright *et al*. (2008) and includes modifications by other authors (Tavares *et al*. 2006, Schweizer *et al*. 2010). This phylogeny encompasses all genera present in this study with the exception of *Psitteuteles*. Previous studies were informative (Christidis *et al*. 1991, Schweizer *et al*. 2010) but not effective for precisely placing Psitteuteles in my phylogeny so I determined the position of *Psitteuteles* relative to *Charmosyna*, *Lorius*, *Chalcopterus*, *Pseudeos*, *Eos* and *Trichoglossus* by reconstructing the clade using a bayesian Markov Chain Monte Carlo approach implemented in MrBayes (Huelsenbeck and Ronquist 2001). Cytochrome b sequences were obtained from Genbank (Benson *et al*. 2005) and aligned in BioEdit (Hall 1999) resulting in a shared sequence 853bp in length. I used a general time reversible Bayesian model run for 10,000 generations and sampled every 10 generations following a burn in of 2000 generations. The resulting tree structure placed *Psitteuteles* in its present position in the phylogeny (Appendix 1) with a posterior probability of 100%.

The phylogeny was further modified to resolve species relationships for the speciose clades for which I had immunological data for by appending known phylogenies for *Chalcopsitta* (Astuti *et al*. 2006), *Amazona* (Russello and Amato 2004) and *Cacatua* (Brown and Toft 1999). Since no phylogeny exists for *Ara*, I constructed one from a 430bp shared fragment of the ribosomal RNA 16s gene (Appendix 2) following the same Bayesian procedure used for *Psitteuteles* but using a burn in of 1600 generations.

Statistical Analyses

All statistical analyses were done using R 2.11 (R Core Team 2008). I assessed model fit using AIC values corrected for small samples sizes (Burnham and Anderson 2002) derived from phylogenetically informed Generalized Least Squares (PGLS) regression models using the R packages nlme (Pinheiro *et al*. 2011) and Ape (Paradis *et al*. 2004). From the parameters measured I identified 5 models comprising a sexual selection model, a pace-of-life model, residual pace-of-life (accounting for body mass), an ecological model and a global model (Table 1). For each statistical model I compared the fit of two evolutionary models. PGLS λ transforms branch lengths by optimizing their length based on trait evolution determined by the phylogeny (Pagel 1999). The λ parameter varies between 0 (phylogenetic independence) and 1 (phylogenetic dependence). The Ornstein-Uhlenbeck process applies a similar branch length transformation however assumes a constraint on trait evolution (Martins and Hansen 1997). Results from these models were similar and did not influence their interpretation, and so all results given are from PGLS λ models. I used the first principal component of all plumage variables as a measure of plumage exaggeration (Table 2). I used phylogenetic PC (Revell 2012) scores obtained using the λ method because using PC scores derived using λ and brownian motion models did not influence the interpretation of the results.

Results

The ISIS dataset included 66 species of parrot ranging from 3-119 individuals per species (median: 12 individuals per species). Across species, heterophils comprised 53% of all leukocytes followed by lymphocytes (37%), monocytes (5%), eosinophils (3%) and basophils (2%). Initial investigation revealed two outliers (>3.6 standard deviations) for total leukocyte counts. The cause of this was extremely high heterophil counts (4.5 and 5.5 standard deviations

Table 1. Life history parameters incorporated into five models used to explain leukocyte concentration.

	Sexual Selection	Pace-of-Life	Residual Pace-of-Life	Ecological	Global
Plumage Brightness	Y				Y
Sexual Dichromatism	Y				Y
Sexual Size Dimorphism	Y				Y
Incubation Period		Y	Y		Y
Clutch Size		Y	Y		Y
Longevity		Y	Y		Y
Log[Mass]			Y		Y
Diet				Y	Y
Region				Y	Y
Habitat				Y	Y
Flock Size				Y	Y

Table 2. Eigenvectors for plumage traits from a phylogenetically informed PCA.

Plumage Trait	PC1
Brightness	0.82
Contrast	0.85
Complexity	0.70
Non-background	0.80
Non-green	0.51
Red/Orange/Yellow	0.88

above mean counts). These outliers had a strong effect on subsequent models and were identified as highly influential based on Cook's distance values derived from residual-leverage plots. These two species were two of three species from the genus *Chalcopsitta*. The third species also had very high leukocyte counts but were within the range expected for all species. However, its high leukocytes were again driven by extremely high heterophil counts (3.4 standard deviations). Because all members of *Chalcopsitta* in this study show these extreme counts they appear to be evolutionarily derived but it is not clear what aspect of their life history accounts for selection for such high counts. Because of the influence of these extreme data points all three were excluded

from analyses involving heterophils, and the former two were excluded from analyses of leukocytes.

Correlations among the five leukocyte types were generally low. The strongest relationships were between heterophils and eosinophils (r=0.29, P=0.02) and between lymphocytes and monocytes (r=0.36, P=0.004), which represents natural groupings of granulocytic and non-granulocytic leukocytes. All cell types increased with total leukocyte count, but the strength of the relationship was proportional to the abundance of the cell type.

Table 3. The relationship between aspects of leukocytes and plumage colouration from bivariate analyses. In general, species with more elaborate, but not more complex, plumage had higher leukocyte counts. λ values were tested against null models (λ=0, complete phylogenetic independence) or phylogenetic dependence (λ=1) using likelihood ratio tests. Significant differences from 0 (first position) and 1 (second position) are denoted by *. Eosinophil counts were not available for one species. See Results for sample size explanations otherwise.

Predictor	Trait	n	Λ	AICc	t	P
Total Leukocytes	PC Plumage	64	$0.05^{ns,*}$	310.58	2.67	**0.01**
	Red/Orange/Yellow	64	$0.04^{ns,*}$	303.46	3.61	**<0.001**
	Brightness	64	$0.04^{ns,*}$	303.56	3.32	**0.002**
	Contrast	64	$0.02^{ns,*}$	309.73	2.04	**0.05**
	Complexity	64	$0.17^{ns,*}$	311.81	0.91	0.37
	Non-background	64	$0.18^{ns,*}$	317.81	1.05	0.30
	Non-green	64	$0.17^{ns,*}$	318.58	1.24	0.22
Heterophils	PC Plumage	63	$0.41^{*,*}$	259.56	2.22	**0.03**
Lymphocytes	PC Plumage	64	$0.50^{*,*}$	235.77	2.38	**0.02**
Eosinophils	PC Plumage	63	$0.02^{ns,*}$	-2.64	-0.24	0.81
Monocytes	PC Plumage	64	$0.18^{ns,*}$	11.19	-0.20	0.84
Basophils	PC Plumage	64	$0.10^{ns,*}$	-84.52	2.24	**0.03**

Species with more exaggerated plumage traits had higher leukocyte counts (Table 3). This was especially the case for overall brightness and the extent of red, orange and yellow based plumage, and to a lesser extent the degree of contrast. On the other hand, aspects complexity, including the amount of non-background colouration was not related to leukocytes. Among leukocyte types, heterophils, lymphocytes and basophils were positively related to plumage colour but eosinophils and monocytes were not related (Table 3).

Some data were not available for all species and so the comparison of models presented in Table 1 were done on a reduced dataset (n=45). The sexual selection model performed the best of all models (Table 4). PC plumage colour (t=3.09, P=0.004) was positively related to leukocyte count whereas sexual dichromatism was negatively related (t=-3.28, P=0.002). The degree of sexual size dimorphism was not related to leukocytes. The remaining models achieved relatively little support. Individual variables included in those models were poor predictors of leukocyte counts with a couple of exceptions. In the ecological model, species associating in larger flocks had fewer leukocytes (t=-2.38, P=0.02; all other variables P>0.17). Generally, pace-of-life models performed poorly but the length of the incubation period was positively related to leukocytes (residual pace-of-life model: t=2.30, P=0.03; pace-of-life model: t=2.45, P=0.02; all other variables P>0.66 for both models). Plumage colour (t=3.03, P=0.005) was the only variable that remained significant in the in the global model although flock size (t=-1.79, P=0.08) and incubation period (t=1.79, P=0.08) approached significance.

Table 4. Performance of selected models explaining total leukocyte counts. Models were run with a subset of species (n=45) for which all data were available.

Model	λ	K	AICc	ΔAICc	*L*ΔAICc	AICc*W*	Evidence Ratio
Sexual Selection	$-0.16^{ns,*}$	3	207.387	0.000	1.000	0.991	1.000
Ecological	$-0.16^{ns,*}$	4	216.877	9.490	0.009	0.009	115.033
Pace-of-Life	$0.24^{ns,*}$	3	222.955	15.568	0.000	0.000	2402.103
Residual POL	$0.25^{*,*}$	4	236.316	28.929	0.000	0.000	1913739.990
Global	$0.15^{ns,*}$	11	239.387	32.000	0.000	0.000	8887621.288

Discussion

Stronger Sexual Selection has both a positive and negative effect on Immune Investment.

Plumage colour was positively related to leukocyte counts and was the strongest single variable predicting leukocyte counts in any model. Similarly, the sexual selection model performed better than the pace-of-life and ecological models. Although I was not able to compare infection rates in this study, others have shown that brighter species tend to have higher rates of infection (Hamilton and Zuk 1982], Read 1987, Scheurlein and Ricklefs 2004, but see Read and Harvey 1989). These findings reinforce the idea that selection for brighter, more immuno-competent individuals within species results in greater species level immune investment. Moreover, bearing these elaborate traits does not result in a cost imposed on the immune system as could result from an immuno-competence handicap type mechanism (Folstad and Karter 1992).

By contrast, immune investment was lower in species with marked sexual dichromatism. This is consistent with studies showing greater mortality in dichromatic species (Promislow *et al*. 1992), but is contrary to studies demonstrating a positive relationship between dichromatism and spleen size (Møller 1997, Møller *et al*. 1998). Cavity nesting, which occurs in all parrots in some form (del Hoyo *et al*. 1997), liberates incubating females from selection on crypsis and so sexual dichromatism is less frequent in cavity nesting species (Scott and Clutton-Brock 1990). This

means that expression of plumage colouration can be similar in males and females with species brightness reflecting selection for, but not constraint on, plumage colouration. Sexual dichromatism more frequently occurs due to selection for reduced ornamentation in one sex (Badyaev and Hill 2003). Yet, there is no obvious cause for dichromatism in the species of parrots in my sample. Female crypsis should occur in species where nests are more exposed but dichromatic species in this study do not use more open nesting hollows versus cavities nor is there an interaction with plumage brightness.

Sexual size dimorphism was not related to leukocytes. However, size dimorphism may not be an accurate measure if it is more detectable in larger species. If this is the case, it would result in biased estimates of the degree of dimorphism when grouping birds across a large range of body sizes. I addressed this by analyzing those species less than 120g, and separately only large bodied species (>415g), and there was still no effect of sexual size dimorphism on leukocytes in either case. In most birds, sexual size dimorphism results from divergent sex roles (Fairbarin *et al*. 2007), but parrots are largely monogamous, with males feeding females throughout the incubation period. This likely explains why sexual size dimorphism is limited in parrots, with fewer than 40% of the species in this study exhibiting some level of size dimorphism. It would be insightful to understand the interplay between sexual size dimorphism, sexual dichromatism and plumage colouration. However, it is clear that as a measure of the intensity of sexual selection, sexual size dimorphism does not explain investment in the immune system in parrots.

Taken together, leukocyte counts were positively related to overall plumage expression, yet negatively related to the degree of sexual dichromatism. Thus, absolute trait expression and the degree of difference in trait expression between the sexes had opposing effects on immunity.

Costs associated with bearing elaborate SSC's, such as reduced immunity and/or higher mortality across species, may not be a function of the trait per sé but rather behaviours associated with large sexual dimorphisms since sexual dimorphisms result from different selective trajectories for males and females (Arnqvist and Rowe 2005). For example, higher costs in more dimorphic species (Promislow 1992, Moore and Wilson 2002, Promislow *et al*. 1994, Nunn *et al*. 2009) may stem from behaviours associated with more elaborate sexual traits and divergent behaviours such as courtship, territoriality, or other energetic trade-offs independent of specific costs of the traits being measured.

Longevity, Pace-of-life and Immunity

Both longevity and residual longevity proved to be surprisingly poor predictors of investment in immunity. Lifespan and immunity are expected to be positively related either because a competent immune system enables long life, or because species with genetically determined long lives preferentially invest in immunity to achieve their maximum lifespan potential. In the few cases where lifespan has been used in comparative studies, it was not related to cell-mediated immunity in birds (Tella *et al*. 2002), but was related to leukocyte counts in mammals, although the result was limited to females (Nunn *et al*. 2009). As expected though, longevity was predictably correlated with pace-of-life traits (incubation period $r=0.41$, $P=0.005$; clutch size $r=-0.38$, $P=0.009$) and the relationship between body size and longevity was strong ($r=0.52$, $P<0.001$) and consistent with what is typically reported in birds (Hulbert *et al*. 2007, de Magalhaes *et al*. 2007).

Some have argued that MLSP is not as biologically relevant as longevity in the wild (Monoghan and Metcalfe 2001, but see Ricklefs 2000) possibly because specific traits, such as those associated with pace-of-life, are likely the proximate determinants of trade-offs with other

life history traits, with shorter MLSP simply being a by-product of increased emphasis on reproduction. In the present study, pace-of-life models received relatively little empirical support based on AIC values, however, incubation period was positively related to leukocyte counts. This result is consistent with pace-of-life studies, which frequently show relationships between immune investment and incubation period but not with other pace-of-life traits, such as clutch size (Tella *et al*. 2002, Palacios and Martin 2006, Lee *et al*. 2008).

Species with long incubation periods typically have young that take longer to fledge and generally slower developmental rates. Longer developmental periods in the nest inherently expose offspring to an increased risk of nest-born parasites, something that could be especially problematic for cavity nesting species that tend to reuse nest sites. Alternatively, slow development may delay maturation of the acquired immune response which increases selection on innate aspects of immunity (Lee 2006). This may be the case because among the pace-of-life variables used in the present study, only incubation period was related to leukocyte counts. On the other hand, increased maternal antibody transfer does not seem adequate to offset the delayed maturation of the acquired immune system in slow paced species (Addison *et al*. 2009), although it has been suggested that persistence of these antibodies might be greater in species with slower pace-of-life (Garnier *et al*. 2012).

More gregarious species have fewer leukocytes.

Generally, the ecological model did not explain immune investment. However, I found that parrot species that form larger flocks have fewer leukocytes. This seems counterintuitive since the risk of infection should increase at higher densities (Côté and Poulin 1995) and in support of this, immune investment is often higher among animals in larger groups (Møller *et al*. 2001, Nunn 2002, Nunn *et al*. 2003). Yet several studies, including this one, report the opposite pattern

(e.g., Lee *et al*. 2008, Watve and Sukumar 1997), which has led to the hypothesis that in large groups, individuals may benefit from a host dilution effect (Wilson *et al*. 2003, Watve and Jog 1997). This may be an appropriate explanation for parrots because flocks are often temporally or spatially ephemeral (del Hoyo *et al*. 1997) which may limit the ability for vectors to track host aggregations.

A note on Red-, Orange- and Yellow-based Colouration in Parrots.

Most plumage traits were positively correlated amongst themselves but brightness, contrast and red/orange/yellow were the most important variables comprising the first principal component of plumage colour (Table 2). Not surprisingly, the degree of red, orange and yellow colouration was strongly related to leukocyte count. Unlike in passerines where these colours are produced by dietary carotenoids, in parrots the colours result from a less studied class of non-dietary pigments called the psittacofulvins (Stradi *et al*. 2001). The present study evidences a link between the immune system and psittacofulvins. Psittacofulvin-derived plumage traits have been shown to be condition-dependent (Masello *et al*. 2008), and psittacofulvins themselves have antioxidant properties (Morelli *et al*. 2003, Pini *et al*. 2004), and so likely signal similar physiological information to carotenoid-based traits. However, it is noteworthy that parrots use psittacolfulvins in lieu of carotenoids despite having adequate plasma carotenoid levels (McGraw and Nogare 2005). Understanding the relative costs and the contributions to physiological processes of these two pigments is an interesting avenue for future research.

The complexity of the immune system cannot be adequately described using a single measure, and because of this the results from this study should be considered accordingly. Inconsistencies across comparative studies can be attributed, in part, to individual immune measures being related to life history traits in unique ways. I used leukocyte counts as a means of

assessing immune investment. Although this measure has been considered unreliable for wild species (Hasselquist 2007), circulating leukocytes are regularly used to assess health and immunocompetence in both captive animals and humans.

The current comparative study adds to our knowledge of the role the immune system plays in the relationship between plumage brightness and parasite loads. Arguably, high parasite loads in brighter species could be the result of poor investment in immunity, perhaps as a consequence of investment in costly SSC's or other aspects of reproduction. By contrast, the results from the present study are consistent with intra-sexual selection producing brighter and more immuno-competent individuals, and that elaborate plumage need not be a handicap as proposed by the ICHH. I did note a negative effect of sexual dichromatism, which suggests the need to control for divergent sexual strategies when studying the costliness of SSC's.
Similar to other recent studies (Tella *et al*. 2002, Nunn *et al*. 2009), longer lived species did not invest more in leukocytes, however species with slower pace-of-life did. Pace-of-life traits may be a better measure of immune investment than longevity, and in particular, immune investment may be related costs associated with long developmental periods.

Literature Cited

Addison, B., K.C. Klasing, W.D. Robinson, S.H. Austin and R.E. Ricklefs. 2009. Ecological and life-history factors influencing the evolution of maternal antibody allocation: a phylogenetic comparison. Proc Biol Sci 276: 3979-3987.

Alderton, D. 2000. The Ultimate Encyclopedia of Caged and Aviary Birds. New York: Lorenz Books. 256 p.

Anderson, M.J., J.K. Hessel and A.F. Dixson. 2004. Primate mating systems and the evolution of immune responses. J Reprod Immunol 61: 31-38.

Andersson, M. 1994. Sexual Selection. Princeton: Princeton University Press. 624p.

Arnqvist, G. and L. Rowe. 2005. Sexual Conflict. Princeton: Princeton University Press. 330p.

Astuti, D., N. Aruma, H. Suzuki and S. Higashi. 2006. Phylogenetic relationships within parrots (Psittacidae) inferred from mitochondrial Cytochrome-b gene sequences. Zoolog Sci 23: 191-198.

Badyaev, A.V. and G.E. Hill. 2003. Avian sexual dichromatism in relation to phylogeny and ecology. Annu Rev Ecol Evol Syst 34: 27-49.

Benson, D.A., I. Karsch-Mizrachi, D.J. Lipman, J. Ostell and D.L. Wheeler. 2005. GenBank. Nucleic Acids Res 33: D34-D38.

Bonduriansky, R., A. Maklakov, F. Zajitschek and R. Brooks. 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. Funct Ecol 22: 443-453.

Brouwer, K., M.L. Jones, C.E. King and H. Schifter. 2000. Longevity records for Psittaciformes in captivity. International Zoo Yearbook 37: 299-316.

Brown, D.M. and C.A. Toft. 1999. Molecular systematics and biogeography of the cockatoos (Psittaciformes: Cacatuidae). Auk 116: 141-157.

Burnham, K.P. and D.R. Anderson. 2002. Model selection and mulitmodel inference: A practical information-theoretic approach. New York: Springer Verlag. 488 p.

Catoni, C., H.M. Schaefer and A. Peters. 2008. Fruit for health: the effect of flavonoids on humoral immune response and food selection in a frugivorous bird. Funct Ecol 22: 649-654.

Christidis, L., R. Schodde, D.D. Shaw and S.F. Maynes. 1991. Relationships among the Australo-Papuan Parrots, Lorikeets and Cockatoos (Aves: Psittaciformes): Protein evidence. Condor 93: 302-317.

Côté, I.M. and R. Poulin. 1995. Parasitism and group size in animals: a meta-analysis. Behav Ecol 6: 159-165.

de Magalhaes, J.P., J. Costa and G.M. Church. 2007. An analysis of the relationship between metabolism, developmental schedules, and longevity using phylogenetic independent contrasts. J Gerontol 62A: 149–160.

del Hoyo, J., A. Elliot and J. Sargatal. 1997. Handbook of the Birds of the World. Volume 4: Sandgrouse to Cuckoos. Barcelona: Lynx Edicions. 600p.

Dunning, J.B. 2008. CRC handbook of avian body masses, 2nd ed. Boca Raton: Taylor and Francis Group. 672 p.

Endler, J.A. 1993. The color of light in forests and its implications. Ecol Monogr 63: 1-27.

Fairbairn, D.J., W.U. Blanckenhorn and T. Székely. 2007. Sex, size and gender roles: Evolutionary studies of sexual size dimorphism. Oxford: Oxford University Press Online.

Folstad, I. and A.J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603-622.

Forshaw, J.M. 1973. Parrots of the World. New York: Doubleday. 584 p.

Garnier, R., R. Ramos, V. Staszewski, T. Militao, E. Lobato, J. González-Solís and T. Boulinier. 2012. Maternal antibody persistence: a neglected life-history trait with implications from albatross conservation to comparative immunology. Proc Biol Sci 279: 2033-2041.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Hamilton, W.D. and M. Zuk. 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Hasselquist, D. 2007. Comparative immunoecology in birds: hypotheses and tests. J Ornithol 148: S571-S582.

Holmes, D.J. and S.N. Austad. 1995. The evolution of avian senescence patterns: implications for understanding primary aging processes. Am Zool 35: 307-317.

Hulbert, A.J., R. Pamplona, R. Buffenstein and W.A. Buttemer. 2007. Life and death: metabolic rate, membrane composition and life span of animals. Physiol Rev 87: 1175–1213.

Juniper, T. and M. Parr. 1998. Parrots: a guide to parrots of the world. New Haven: Yale University Press. 584 p.

Lee, K.A. 2006. Linking immune defenses and life history at the levels of the individual and the species. Integr Comp Biol 46: 1000-1015.

Lee, K.A., M. Wikelski, W.D. Robinson, T.R. Robinson and K.C. Klasing. 2008. Constitutive immune defences correlate with life-history variables in tropical birds. J Anim Ecol 77: 356-363.

Lochmiller, R.L. and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: Just what is the cost of immunity? Oikos 88: 87-98.

Low, R. 1998. Encyclopedia of Lories. Surrey: Hancock House. 432 p.

Martin, L.B., A. Scheuerlein and M. Wikelski. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc Biol Sci 270: 153-158.

Martins, E.P. and T.F. Hansen. 1997. Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. Am Nat 149: 646-667.

Masello, J.F., T. Lubjuhn and P. Quillfeldt. 2008. Is the structural and psittacofulvin-based coloration of wild burrowing parrots *Cyanoliseus patagonus* condition-dependent? J Avian Biol 39: 653-662.

Matson, K.D. 2006. Are there differences in immune function between continental and insular birds? Proc Biol Sci 273: 2267-2274.

Mayr, G. 2010. Parrot interrelationships – morphology and the new molecular phylogenies. Emu 110: 348-357.

McGraw, K.J. and M.C. Nogare. 2005. Distribution of unique red feather pigments in parrots. Biol Lett 1: 38-43.

McNaught, M.K. and I.P.F. Owens. 2002. Interspecific variation in plumage colour among birds: species recognition or light environment. J Evol Biol 15: 505-514.

Møller, A.P. 1997. Immune defence, extra-pair paternity, and sexual selection. Proc Biol Sci 264: 561-566.

Møller, A.P. 1998. Host immune defence and migration in birds. Evol Ecol 12:945-953.

Møller, A.P. and J. Erritzøe. 1998. Evidence of larger impact of parasites on hosts in the tropics: investment in immune function within and outside the tropics. Oikos 82: 265-270.

Møller, A.P., R. Dufva and J. Erritzøe. 1998. Host immune function and sexual selection in birds. J Evol Biol 11: 703-719.

Møller, A.P., P. Christe and E. Lux. 1999. Parasitism, host immune function and sexual selection. Q Rev Biol 74: 3-20.

Møller, A.P., S. Merino, C.R. Brown and R.J. Robertson. 2001. Immune defense and host sociality: A comparative study of swallows and martins. Am Nat 158: 136-145.

Monoghan, P. And N.B. Metcalfe. 2001. Genome size, longevity and development time in birds. Trends Genet 17: 568.

Moore, S.L. and K. Wilson. 2002. Parasites as a viability cost of sexual selection in natural populations of mammals. Science 297: 2015-2018.

Morelli, R., R. Loscalzo, R. Stradi, A. Bertelli and M. Falchi. 2003. Evaluation of the antioxidant activity of new carotenoid-like compounds by electron paramagnetic resonance. Drugs Exp Clin Res 29: 95-100.

Munshi-South, J. and G.S. Wilkinson. 2006. Diet influences life span in parrots (Psittaciformes). Auk 123: 108-118.

Norris, K. and M.R. Evans. 2000. Ecological immunology: life history trade-offs and immune defense in birds. Behav Ecol 11: 19-26.

Nunn, C.L. 2002. A comparative study of leukocyte counts and disease risk in primates. Evolution 56: 177-190.

Nunn, C.L., J.L. Gittleman and J. Antonovics. 2000. Promiscuity and the primate immune system. Science 290: 1168-1170.

Nunn, C.L., J.L. Gittleman and J. Antonovics. 2003. A comparative study of white blood cell counts and disease risk in carnivores. Proc Biol Sci 270: 347-356.

Nunn, C.L., P. Lindenfors, E.R. Pursall and J. Rolff. 2009. On sexual dimorphism in immune function. Philos Trans R Soc Lond B Biol Sci 364: 61-69.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. Nature 401: 877–884.

Palacios, M.G. and T.E. Martin. 2006. Incubation period and immune function: a comparative field study among coexisting birds. Oecologia 146: 505-512.

Paradis, E., J. Claude and K. Strimmer. 2004. APE: analysis of phylogenetics and evolution in R language. Bioinformatics 20: 289-290.

Peach, W.J., D.B. Hammer and T.B. Oatley. 2001. Do southern African songbirds live longer than their European counterparts? Oikos 93: 235-249.

Pinheiro. J., D. Bates, S. DebRoy, D. Sarkar the R Development Core Team. 2011. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-98.

Pini, E., A. Bertelli, R. Stradi and M. Falchi. 2004. Biological activity of parrodienes, a new class of polyunsaturated linear aldehydes similar to carotenoids. Drugs Exp Clin Res 30: 203-206.

Promislow, D.E.L. 1992. Costs of sexual selection in natural populations of mammals. Proc Biol Sci 247: 203-210.

Promislow, D.E.L. and P.H. Harvey. 1990. Living fast and dying young: A comparative analysis of life-history variation among mammals. J Zool (1987) 220: 417-437.

Promislow, D.E.L., R. Montgomerie and T.E. Martin. 1992. Mortality costs of sexual dimorphism in birds. Proc Biol Sci 250: 143-150.

Promislow, D.E.L., R. Montgomerie and T.E. Martin. 1994. Sexual selection and survival in North American waterfowl. Evolution 48: 2045-2050.

R Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Råberg, L., J. Nilsson, P. Ilmonen, M. Stjernman and D. Hasselquist. 2000. The cost of an immune response: vaccination reduces parental effort. Ecol Lett 3: 382-386.

Read, A.F. 1987. Comparative evidence supports the Hamilton and Zuk hypothesis on parasites and sexual selection. Nature 328: 68-70.

Read, A.F. and P.H. Harvey. 1989 Reassessment of comparative evidence for Hamilton and Zuk theory on the evolution of secondary sexual characters. Nature 339: 18-620.

Revell, L.J. 2012. phytools: An R package for phylogenetic comparative biology (and other things). Methods Ecol Evol 3: 217-223.

Ricklefs, R.E. 2000. Intrinsic aging-related mortality in birds. J Avian Biol 31: 103-111.

Roitt, I., J. Brostoff and D. Male. 1993. Immunology, 3rd ed. London: Mosby. 420p.

Russello, M.A. and G. Amato. 2004. A molecular phylogeny of Amazona: implications for Neotropical parrot biogeography, taxonomy, and conservation. Mol Phylogenet Evol 30: 421-437.

Scheuerlein, A. and R.E. Ricklefs. 2004. Prevalence of blood parasites in European passeriform birds. Proc Biol Sci 271: 1363-1370.

Schweizer, M., O. Sechausen, M. Güntert and S.T. Hertwig. 2010. The evolutionary diversification of parrots supports a taxon pulse model with multiple trans-oceanic dispersal events and local radiations. Mol Phylogenet Evol 54: 984-994.

Scott, D.K. and T.H. Clutton-Brock. 1990. Mating systems, parasites and plumage dimorphism in waterfowl. Behav Ecol Sociobiol 26: 261-273.

Semple, S., G. Colishaw and P.M. Bennett. 2002. Immune system evolution among anthropoid primates: parasites, injuries and predators. Proc Biol Sci 269: 1031-1037.

Sheldon, B.C. and S. Verhulst. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 11: 317–321.

Stearns, S.C. 1992. The evolution of life histories. Oxford,UK: Oxford University Press. 262p.

Stradi, R., E. Pini and G. Celentano. 2001. The chemical structure of the pigments in *Ara macao* plumage. Comp Biochem Physiol B Biochem Mol Biol 130: 57-63.

Tavares, E.S., A.J. Baker, S.L. Pereira and C.Y. Miyaki. 2006. Phylogenetic relationships and historical biogeography of Neotropical Parrots (Psittaciformes: Psittacidae: Arini) inferred from mitochondrial and nuclear DNA sequences. Syst Biol 55: 454-470.

Tella, J.L., A. Scheuerlein and R.E. Ricklefs. 2002. Is cell-mediated immunity related to the evolution of life-history strategies in birds? Proc Biol Sci 269: 1059-1066.

Tieleman, B.I., J.B. Williams, R.E. Ricklefs and K.C. Klasing. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. Proc Biol Sci 272: 1715-1720.

Valkiunas, G. 2004. Avian malaria parasites and other haemosporidia. Boca Raton: CRC Press, 932 p.

Watve, M.G. and M.M. Jog. 1997. Epidemic diseases and host clustering: an optimum cluster size ensures maximum survival. J Theor Biol 1: 167-171.

Watve, M.G. and R. Sukumar. 1995. Parasite abundance and diversity in mammals correlates with host ecology. Proc Natl Acad Sci USA 92: 8945–8949.

Williams, G.C. 1966. Natural selection, the costs of reproduction, and a refinement of Lack's principle. Am Nat 100: 687–690.

Wilson, K., R. Knell, M. Boots and J. Koch-Osborne. 2003. Group living and investment in immune defence: an interspecific analysis. J Anim Ecol 72: 133–143.

Wright, T.F., E.E. Schirtzinger, T. Matsumoto, J.R. Eberhard, G.R. Graves, J.J. Sanchez, S. Capelli, H. Müller, J. Scharpegge, G.K. Chambers and R.C. Fleischer. 2008. A multilocus molecular phylogeny of the parrots (Psittaciformes): support for a Gondwanan origin during the Cretaceous. Mol Biol Evol 25: 2141-2156.

Zahavi, A. 1975. Mate selection: a selection for a handicap. J Theor Biol 53: 205-214.

Zuk, M., and A.M. Stoehr. 2002. Immune defence and host life history. Am Nat 160: S9-S22.

CHAPTER 3: Linking sex differences in corticosterone with individual reproductive behaviour and hatch success in two species of uniparental shorebirds.

Abstract

In birds, corticosterone (CORT) appears to facilitate reproductive activity because baseline and stress-induced CORT levels are elevated in breeding individuals compared with other times of the year. In particular, CORT is lower in the sex providing most of the parental care (i.e., incubation), which could be an important adaptation to tolerate stressors that result in abandoning reproduction. Therefore, one explanation for sex differences in CORT is that lower levels are favoured during the incubation/parental phase of reproduction. Using two species of uniparental shorebird - polyandrous Red phalaropes (*Phalaropus fulicarius*) and polygynous White-rumped sandpipers (*Calidris fuscicollis*) - I predicted that the incubating sex would have lower baseline and stress-induced CORT, and incubating individuals with lower CORT would more effectively defend nests against a simulated intrusion, would return more quickly afterwards, and would ultimately have higher hatch success. I found that phalaropes followed the predicted pattern: incubating individuals (males) had lower baseline and stress-induced CORT than females but for baseline CORT these differences existed prior to males commencing incubation. Incubating male phalaropes with lower baseline and stress-induced CORT returned to incubate more quickly after a disturbance and there was non-significant tendency for baseline CORT to be lower in successful nests. In sandpipers, I observed no sex differences and no significant relationships between individual CORT levels and nest defence behaviours or hatch success. My results demonstrate that in phalaropes at least, selection favours lower baseline and stress-induced CORT during the nesting period. These results can explain sex differences in stress-induced levels of CORT, however sex differences in baseline CORT were present prior to incubation.

Introduction

Glucocorticoids, such as corticosterone (CORT) in birds, mediate the behavioural and physiological responses to both unpredictable short-term (i.e., acute stressors) and predictable long-term (i.e., life history stages) challenges (Landys *et al*. 2006). As such, individual differences in baseline hormone expression during normal physiological conditions and acute response to stress are thought to be subject to strong selection (Breuner *et al*. 2008, Bonier *et al*. 2009).

At seasonal baseline levels, increases in CORT reflect energetic demands (Bonier *et al*. 2009). Elevated CORT liberates energy stores via gluconeogenesis (Remage-Healey and Romero 2001) and modifies behaviour such as feeding rate (Wingfield *et al*. 1990), aggression (Kitaysky *et al*. 2003) and/or locomotory activity (Wingfield *et al*. 1998). Following exposure to an acute stressor, CORT increases rapidly from baseline levels to initiate behavioural and physiological changes that are thought to promote current survival (Sapolsky *et al*. 2000, Breuner *et al*. 2008); one such effect is to abandon parental duties (e.g., Silverin 1986). Consequently, both baseline (Bonier *et al*. 2009) and acute (Breuner *et al*. 2008) CORT levels are expected to be linked to fitness.

CORT levels typically vary seasonally (Breuner and Orchinik 2001) suggesting that it is adaptively regulated in response to different life history stages, such as breeding. Adrenal activity is upregulated during the breeding season (Romero and Wingfield 1998) and as a consequence, breeding individuals tend to have a higher baseline and acute CORT response to stress (hereafter termed 'stress-induced CORT') than at other times of the year or when compared with non-breeding individuals (Astheimer *et al*. 1994, Romero and Wingfield 1998, Cornelius *et al*. 2012).

Sex differences in CORT are thought to reflect sex-role differences and facilitate sex-specific reproductive behaviours. Among breeding birds, males often have higher baseline and stress-induced CORT than females in species that display sexually dimorphic reproductive behaviours (Astheimer *et al*. 1994, Holberton and Wingfield 2003, Meddle *et al*. 2003). In Tree (*Spizella arborea*) and White-crowned sparrows (*Zonotrichia leucophrys gambelii*), males have higher stress-induced CORT during the preparental stage when behaviours differ between the sexes, but males were similar to females during the parental stage when both sexes fed the young (Holberton and Wingfield 2003). These observations have led to the notion that lower stress-induced CORT in incubating birds functions to prevent nest abandonment by the incubating sex during stressful conditions (O'Reilly and Wingfield 2001). In support of this, the sex providing more parental care has lower stress-induced CORT (males in polyandrous species and females in polygynous species) and there is no apparent sex difference when both sexes contribute evenly (O'Reilly and Wingfield 2001; but see O'Reilly and Wingfield 2003). Furthermore, females have weaker CORT responses in species where they provide more parental care possibly due to the value of the brood as a function of renesting opportunity during shorter breeding seasons (Bókony *et al*. 2009).

Because of these observations, sex differences in CORT are thought to be the result of selection for reduced CORT levels in parental birds. In support of this, breeding adult birds with naturally high levels of baseline CORT have higher abandonment rates (Groscolas *et al*. 2008, Spee *et al*. 2010), as do birds with artificially elevated CORT (Silverin 1986, Angelier *et al*. 2009a, Spee *et al*. 2011). Yet, a natural, acute stress response may be an important part of mounting a strong and successful nest defence. Nest defence is positively related to reproductive success (Garcia 2003, Goławski and Mitrus 2008), and a strong nest defence is especially

important for ground nesting birds where predation accounts for a significant proportion of failed nest attempts and abandonment is otherwise infrequent (Smith and Wilson 2010). Acute glucocorticoid exposure increases aggression in a variety of animals (Wingfield and Silverin 1986, Hayden-Hixson and Ferris 1991, DeNardo and Licht 1993, Haller *et al*. 1997, Kitaysky *et al*. 2003) and so the ability to mount a defence of the nest could be positively related to the magnitude of the adrenocortical response (i.e., stress-induced CORT). However, much of the work to date has been focussed on implants that cause 'short-term' effects that last hours to days, rather than an actual measure of the acute stress response.

As a means of explaining sex differences in CORT levels, I sought to link individual behaviour and hatch success with baseline and stress-induced CORT levels in two species of uniparental shorebird: polygynous White-rumped sandpipers (*Calidris fuscicollis*) and polyandrous Red phalaropes (*Phalaropus fulicarius*). In polygynous sandpipers, females are the sole incubators but in phalaropes males incubate and females do not. I outline three predictions addressed by this study. Prediction 1) If baseline and stress-induced CORT levels are attenuated to facilitate incubation and prevent nest abandonment, I predict that the incubating sex (female sandpipers and male phalaropes) should have lower levels than the non-incubating sex. Prediction 2) Because CORT is expected to be related to defensive and nest guarding behaviour, I predicted that individual incubators with low baseline and stress-induced CORT levels should flush less readily from nests, perform stronger nest defence displays and return more quickly to the nest following a disturbance. Prediction 3) Individuals with lower CORT levels will have higher hatch success.

Methods

Field Site and Species

I examined White-rumped sandpipers (*C. fuscicollis*) and Red phalaropes (*P. fulicarius*) (hereafter referred to as sandpipers and phalaropes) at East Bay Migratory Bird Sanctuary, Nunavut, Canada (N63 59 13.5 W81 41 48.3) during June-July 2008-2010. These two species are small-bodied ground nesting shorebirds. At my site, mean male and female body mass of sandpipers is 43.7g and 42.6g respectively, and for phalaropes 49.4g and 57.1g. During incubation, both species rely on crypsis to avoid nest predation until a predator approaches too closely. Once the predator has approached too closely, the two species employ slightly different tactics: sandpipers undertake rather convincing distraction displays (e.g., 'broken-wing display' or 'rodent run') with relatively few individuals leaving the nest site without producing any display. By contrast, phalaropes frequently flush without producing a display, or less frequently, perform a milder form of a broken-wing display (pers. obs.). The nesting habitats of the two species overlap completely at my field site with most individuals of both species nesting in upland *Dryas* hummocks and sedge meadows; the landscape if predominantly flat with vegetation less than 15cm in height.

Trapping and Blood Collection

I trapped male sandpipers, and both male (prior to incubation) and female phalaropes during the courtship period; I was unable to capture adequate numbers of female sandpipers at this time, and most of those I did capture were carrying eggs and so considered in a physiologically distinct stage. I termed pre-incubation birds 'courtship'. I used the presence of a brood patch to determine whether male phalaropes were incubating. I caught courtship birds by stalking them while they were feeding or displaying/defending territories using a Super Talon Net Gun (Fly Dragon Technology Co., Ltd) (Edwards and Gilchrist 2011). I trapped incubating female

sandpipers and incubating male phalaropes using either bownets or Fundy Jerk Traps placed on the nest. These traps allow birds to come and go from the nest and are manually triggered so can be placed for longer periods of time without interrupting feeding and incubation intervals.

I caught a total of 62 White-rumped sandpipers (45 incubating females and 17 courtship males) and 112 phalaropes (38 courtship females, 37 courtship males and 36 incubating males). Both species are polygamous and courtship activities of male sandpipers and female phalaropes extend into the incubation period. Nevertheless, courtship activity declines making courtship birds more scarce and incubating birds more abundant as the season progresses. Additionally, I could not confirm that birds trapped later in the season were not in an ambiguous or confounding physiological state such as could be expected by including failed or post-breeding individuals in the study. For these reasons, trapping periods did not perfectly overlap for some stages. I trapped male sandpipers from 156 to 173 Julian Day (JD; mean 164) and incubating females from 172 to 204 JD (mean 186). In phalaropes, females were trapped from 161 to 188 JD (mean 171), courtship males 160 to 188 JD (mean 172) and incubating males from 171 to 201 JD (mean 189).

A stopwatch was started immediately when the trap was deployed (either the net gun was shot or the nest trap triggered) and a 'baseline' blood sample was taken within 4 minutes, but usually in less than 3min (90% of samples; mean baseline bleed time: 2.3min ±0.8min). The bird was measured, banded and subjected to a standard stress protocol (Wingfield *et al*. 1992). Blood was collected in heparinised capillary tubes, and volumes drawn were typically between 50 and 100uL per bleed as blood samples were used as part of a larger study. After 30 minutes (mean stress-induced bleed time after first bleed: 30.4min ±1.7min) a second blood sample was taken from the other wing to reflect the 'stress-induced' value for CORT (hereafter, stress-induced CORT). There was no difference between trapping methods in the length of time it took to

collect a blood sample. Blood samples were kept cool for up to 6 hours at which point they were centrifuged and the plasma frozen at -20°C until further analysis. Courtship males and incubating males represented different individuals. All capture and collection protocols were approved by Laurentian University Animal Care Committee and Environment Canada.

Incubating and non-incubating birds were trapped using different methods, which could influence CORT levels. For example, stalking birds could elicit a premature increase in CORT levels, even though birds did not show obvious signs of distress when doing so. To compare the effects of trapping technique, I trapped a subsample (n=9) of incubating male phalaropes in 2010 while they were away from their nests using the net gun (in the same manner as courtship birds). There was no difference in CORT levels between incubating birds caught with the net gun and those caught using traps placed at the nest site (t_{37}=1.75, p=0.20).

Nest Searching and Assessing Hatch Success

I found nests throughout the breeding season during extensive searches of the 3×4km study area, either by observing birds returning to the nest or by flushing incubating birds off the nest. I estimated hatch date using one of two methods in order to confirm the fate of the nest. I could confirm lay date (commencement of incubation) in nests located during the laying sequence and I used the average incubation duration for the species to predict the expected hatch date. When completed clutches were found, I floated the eggs in water to estimate the stage of incubation (Liebezeit *et al*. 2007). I visited nests daily 4 days prior to the estimated hatch date to account for error in hatch estimates but increased the frequency of visits as hatch date drew near. I used hatch success as a measure of reproductive success because chicks typically leave the nest after 24hrs of hatch (Colwell 2010) and are difficult to track once they do.

Nest Defence Behaviours

I measured four aspects of nest defence behaviours in response to a human approaching the nest while the birds were incubating. These trials were conducted during the second visit to the nest for all nests whose clutches were completed when found, and either the second or third visit for those nests that were initially found during the laying sequence. As a means to correct for variation in nest defence behaviours, nests were approached from upwind following a clear line of sight to the nest to ensure that the observer was in full view of the incubating bird.

First, I measured 'primary flush distance', which was the distance between the observer and the bird when it first flushed from the nest. In the 2 minutes after being flushed, I estimated the closest distance between the bird and the observer, termed 'secondary distance'. When the trial was complete, primary flush and secondary distances were measured by either pacing over short distances (<10m) or by handheld GPS unit (>10m) to points marked during the trial. Upon flushing the bird off the nest, I categorically defined their subsequent behaviour as: 1) the bird left the area without performing nest defence behaviours, 2) performed either a 'rodent-run' or 'broken-wing display', 3) performed an escalated version of the previous behaviour that included calling, 4) the bird performed short hovering flights near the observer to attract attention, in conjunction with calls and other behavioural displays. Finally, I recorded 'return time' as the length of time the bird took to return to incubate after the disturbance. To estimate return time, the observer would withdraw to about 75-100m from the nest and observe the parent with binoculars for up to 15min. The distance from which I observed the nest varied due to topography, and for a small number of nests I could not get accurate measurements of return time because I lost sight of the bird. I limited the observation to 15 minutes in case my presence prevented the bird from returning. However, all but one bird had returned before 15 minutes, and

birds typically were no longer displaying nest defence behaviours once I were more than 30m from the nest upon leaving the nest area.

I collected wind speed using a handheld wind metre (Dwyer Instruments, Michigan City, IN, USA). Cloud cover was categorized as either overcast including broken skies or clear, including scattered clouds. Nests were not disturbed during high winds (>30km/h at ground level) or during precipitation. A centrally located weather station (within 3.5km of nests) recorded the daytime low temperature.

In order to obtain blood samples that were closely linked to nest defence behaviours, I deployed traps on the nests during the nest visit where I collected the behavioural measures. I returned to trigger the traps 4-6 hours after their placement to allow birds to calm after the previous visit. While traps were deployed, all birds returned to incubate and no nests were lost to predators.

Laboratory Analyses

Plasma corticosterone levels were measured in duplicate with a double antibody radioimmunoassay (MP Biomedicals, Orangeburg, New York) as per previously published protocols (Washburn *et al*. 2002). The CORT antibody has low cross-reactivity with cortisol (0.05%), deoxycorticosterone (0.34%), aldosterone (0.03%) and 17ß-E_2 (<0.01%). Briefly, the assay was conducted following the manufacturer's directions, except that the volumes of the reagents were halved and the plasma was diluted (5µL of plasma plus 245µL of assay buffer) for baseline samples, and (2.5µL plasma plus 247.5µL of assay buffer) for stress-induced samples. Intra-assay variation was 7.2%. The inter-assay variation was 15.6% (n=15 assays) and the lowest point on the standard curve was 3.125pg CORT/tube.

Male sandpipers were targeted based on their territorial behaviour or appearance (i.e., enlarged vocal sacs or small body size), or sexed in the hand by the presence of a cloacal protuberance. However, I could not be certain of the correct sex in all cases and so sex was corroborated genetically in non-incubating sandpipers following standard procedures using P2/P8 (Griffiths *et al*. 1998) and 2550F/2718R primers (Fridolfsson and Ellegren 1999). DNA was extracted from blood by heating samples at 95°C in a 5% Chelex 100 suspension and results were visualized on 3% agarose gels.

Statistical Analyses

I tested for sex differences in baseline and stress-induced CORT, as well as their effect on nest defence behaviours and hatch success, using linear models. I aim to determine whether sex differences exist between males and females, and ultimately whether these can be explained by selection through incubation activities as has been previously hypothesized (O'Reilly and Wingfield 2001). Because female phalaropes and male sandpipers do not incubate, I could not make comparisons between the sexes at this stage. I tested for sex differences in CORT separately for each species, because for phalaropes I had three groups to compare between, while in sandpipers I had two. I used Tukey post hoc tests to determine differences among groups in phalaropes.

Baseline CORT was related to the time to first bleed in phalaropes ($t_{1,22}$=3.62, P=0.002) but not sandpipers ($t_{1,40}$=0.02, P=0.98) despite there being no difference in the average time of first bleed between species. The effect was still significant when using only those phalaropes sampled within 3min (n=101). The time to the second bleed was not related to stress-induced hormone values for either species. Excluding individuals with first bleed times over 3min did not

change the interpretation of the results in sex analyses, or relations between CORT and behaviours or hatch success.

I identified nine variables that could influence both baseline and stress-induced CORT levels: date of trapping, time of trapping, time to first and second bleeds (above), nest age, wind speed, cloud cover, daily low temperature and year. To reduce the potential number of variables included in models, I used hierarchical partitioning to highlight potential covariates that explain corticosterone levels using the hier.part package in the program R (Walsh and Mac Nally 2013). I also initially included assay run as a fixed effect in linear models, however, because including assay run in analyses did not change the conclusions, results are presented without assay run included. Analyses of CORT values on nest defence behaviours and hatch success were done independently for each species because hierarchical partitioning identified a different group of variables for each species.

I explored flush variables using Principal Components Analysis (PCA) to collapse the four flush variables. I square root transformed sandpiper CORT values because they were not normality distributed. In some cases outliers were still present but their exclusion did not alter the results and so they were retained for the analysis. All analyses were completed in R 2.13.1 (R Core Team 2008).

Results

Hierarchical Partitioning

Hierarchical partitioning revealed that time of day had substantial explanatory power for both baseline and stress-induced corticosterone levels in sandpipers and so was included in the analysis. In phalaropes, timing to the first bleed (time between capture and when the first blood sample was taken) had the greatest explanatory power of baseline corticosterone, and along with

time of day and year, was included in the model with baseline values. All variables had poor explanatory power for stress-induced corticosterone in phalaropes.

Prediction 1: Sex Differences in CORT

I began by determining whether CORT levels differed by sex or breeding stage for the two species. There were no sex differences in baseline CORT in sandpipers (Fig. 1A; $F_{1,63}$=0.09, P=0.76) but incubating females had higher stress-induced CORT levels than courtship males (Fig. 1B; $F_{1,61}$=10.52, P=0.002). In phalaropes, males had lower baseline CORT levels in a model including elapsed time of first bleed, year and time of day (Fig. 2A; sex/breeding stage: $F_{2,107}$=9.17, P<0.001) with the most extreme difference between courtship females and incubating males. Pre-incubating males were higher but not statistically distinguishable from incubating males (Tukey: P=0.31). In a reduced dataset including only the birds caught within the period of overlapping trap dates, the effect was still present but marginally significant ($F_{2,49}$=3.05, P=0.056); females had the highest levels of baseline CORT, whereas there was no distinction between courtship and incubating males. A similar pattern was present across females, courtship males and incubating males for stress-induced CORT levels (Fig. 2B; $F_{2,109}$=20.14, P<0.001) although the difference between courtship males and females was marginal (Tukey: P=0.06). When I once again restricted the analysis to only those birds caught within the period of overlapping dates the pattern was similar as before ($F_{2,54}$=11.72, P<0.001), however courtship males changed to being statistically different from females but indistinguishable from incubating males (Tukey: P=0.20).

Prediction 2: Nest Defence Traits and Parental CORT Levels

The results from the PCA were similar for both species (Table 5). The first two components were retained for both species and PC1 explained 54.1% and 41.8% of the variation for sandpipers and

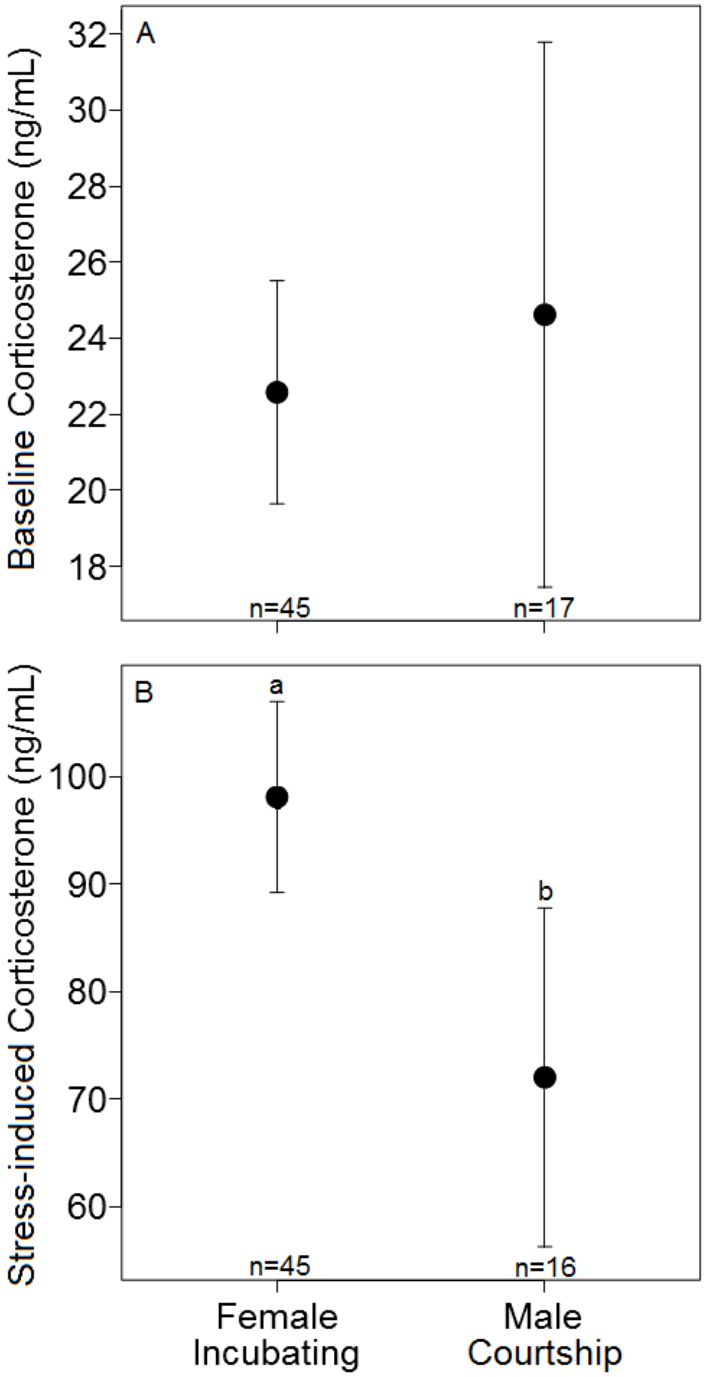


Fig. 1. Sex differences in baseline (A) and stress-induced (B) corticosterone levels among breeding male and incubating female White-rumped sandpipers. Males were trapped during courtship and females were trapped while incubating. Lower case letters denote significant differences (α=0.05) using ANOVA. Bars represent 95% confidence limits.

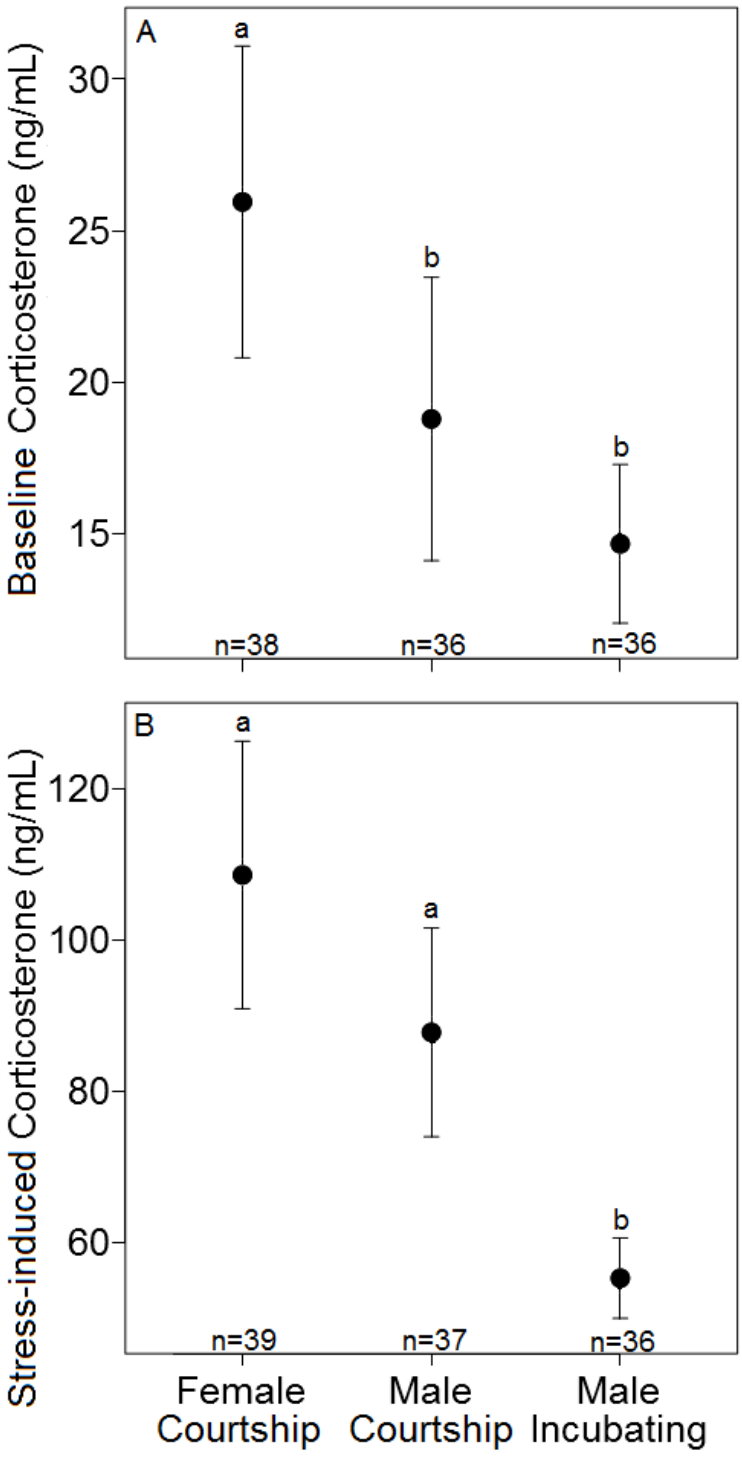


Fig. 2. Sex differences in baseline (A) and stress-induced (B) corticosterone levels among female Red phalaropes and males caught during courtship and incubation stages. Lower case letters denote significant differences (α=0.05) using ANOVA. Bars represent 95% confidence limits.

Table 5. Loadings for nest defence behaviours from principal component analysis.

	WRSA		REPH	
	PC1	PC2	PC1	PC2
Flush Distance	0.57	-0.09	0.49	-0.48
Secondary Distance	0.60	0.11	0.61	0.09
Behaviour	-0.55	0.26	-0.62	-0.31
Return Time	0.13	0.96	-0.01	0.82

phalaropes respectively and PC2 explained 25.7 and 30.7. Flush distance, secondary distance and behaviour loaded heavily on PC1 such that birds with high scores were those with a close flush distance, a close secondary distance and a strong behavioural display. For both species, PC2 was composed primarily of return time but for phalaropes flush distance also contributed. Thus, a bird's tendency to return quickly to the nest following a disturbance was independent of the intensity to which it defended its nest.

PC1, composed of close flush distance, close secondary distance and elevated defensive behaviours, was not related to baseline or stress-induced CORT in either species (Table 6). PC2, composed primarily of return time, was positively related, albeit not significantly so (P=0.09), to baseline CORT in female sandpipers (Table 6). In male phalaropes, PC2 was positively related to both baseline and stress-induced CORT (Table 6).

Because CORT can be related to defence behaviours through body condition, I repeated these models including body mass and tarsus length as factors. The significant factors in the previous models remained significant with the inclusion of these terms suggesting that individual differences in condition contributed little to the effect. In sandpipers, the relationship between

Table 6. Results of linear models used to test for relationships between baseline and stress-induced corticosterone levels and reproductive variables. Nest defence behaviours were included in models as principal component scores (PC; see Table 5.).

Species	N		Model		SE	t	P
White-rumped sandpiper	39	Baseline	*PC1 + PC2 + Capturetime*	PC1	0.12	0.82	0.42
				PC2	0.18	2.00	**0.05**
	32		*Hatchsuccess + Capturetime*	Hatch	0.54	-0.15	0.88
	39	Stress-induced	*PC1 + PC2 + Capturetime*	PC1	0.18	0.27	0.79
				PC2	0.25	-1.03	0.31
	32		*Hatchsuccess + Capturetime*	Hatch	0.68	-0.42	0.68
Red phalarope	17	Baseline	*PC1 + PC2 + Bleedtime + Capturetime + Yr*	PC1	0.96	-0.15	0.88
				PC2	0.92	3.29	**0.007**
	17		*Hatchsuccess + Bleedtime + Capturetime + Yr*	Hatch	2.48	-1.99	0.07
	18	Stress-induced	*PC1 + PC2*	PC1	2.73	0.52	0.61
				PC2	2.60	2.16	**0.05**
	18		*Hatchsuccess*	Hatch	5.68	-1.28	0.22

CORT and body condition, which I interpreted as the partial r of body mass from the previous models, was negligible for baseline ($r_{partial}$=0.09) and stress-induced CORT ($r_{partial}$=-0.01). In phalaropes, the relationships were stronger for both baseline ($r_{partial}$=-0.22) and stress-induced CORT ($r_{partial}$=0.25).

Prediction 3: Hatch Success and Parental CORT Levels

Hatching success was not related to either baseline or stress-induced CORT levels in sandpipers (Table 6; Fig. 3). In phalaropes, there was a non-significant tendency for both baseline and stress-induced CORT to be lower in successful phalaropes (Table 6; Fig. 3). To determine whether the inability to detect an effect in phalaropes was the result of low statistical power, I conducted a post hoc power analysis on the main effect (hatch success) using parameters specified from the previous linear models. Statistical power was low to detect an effect in baseline CORT (power=0.45; recommended level=0.80) and poor for stress-induced CORT (power=0.16).

Table 7. Summary of the results highlighting whether the predictions were supported for baseline/stress-induced CORT. With similar predictions for both baseline and stress-induced CORT, I predicted that [1] the incubating sex has lower CORT, [2] individuals with lower CORT have stronger nest defence, and [3] individuals with lower CORT have higher hatch success. For the purposes of this table, I use only time to return to the nest (PC2) as the sole measure of nest defence, since PC1 did not support any of my predictions (see text).

Species	Incubating	Prediction1		Prediction 2		Prediction 3	
	Sex	Baseline	Stress-induced	Baseline	Stress-induced	Baseline	Stress-induced
White-rumped sandpiper	Female	No	No	No	No	No	No
Red phalarope	Male	Partial	Yes	Yes	Yes	Weak	No

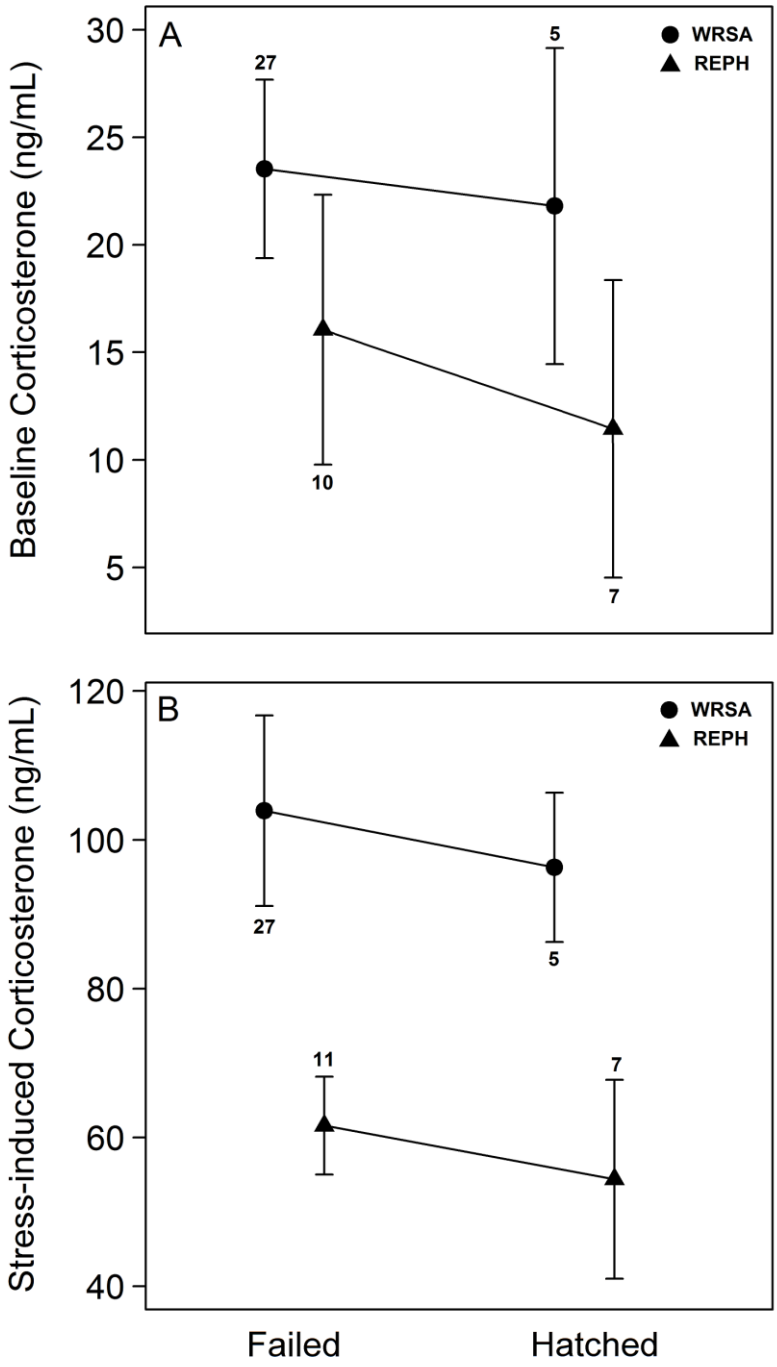


Fig. 3. Baseline (A) and stress-induced (B) CORT levels in incubating White-rumped sandpipers (WRSA, circles) and Red phalaropes (REPH, triangles) with regard to nest fate. The relationship was not significant (ANOVA) for baseline CORT although there was a non-significant tendency for CORT to be lower phalaropes that incubated nests that hatched successfully. Stress-induced levels were higher in failed nesters for both species. Bars represent 95% confidence limits and sample sizes are included for each group.

Discussion

I noted marked species differences in the relationships among CORT and sex and nesting behaviours despite both species being sampled under the same environmental conditions and over the same period of time. Two predictions were supported in phalaropes (where males incubate; Table 7) with weak support for the third, while none were supported in sandpipers (in which females incubate).

Prediction 1: Sex Differences in CORT

Because selection should favour tolerance to stressors during incubation, I sought to establish whether the incubating sex had lower CORT levels in the two species. I expected baseline and stress-induced CORT levels to be lower in female sandpipers and male phalaropes. In sandpipers, I could not compare directly a change within females from the courtship period to incubation because I was not able to catch an adequate number of females during the courtship period. However, if CORT levels are attenuated during incubation, I should see the most distinct differences between courtship males and incubating females. However, this was not the case for baseline CORT, and incubating females actually had higher stress-induced CORT. This result is similar to another species in the same genus, Western sandpipers (*Calidris mauri*), where males and females share incubation duties (O'Reilly and Wingfield 2001, O'Reilly and Wingfield 2003). In fact, the actual baseline and stress-induced CORT values for male and female White-rumped sandpipers I report in my study are very similar to the monogamous Western sandpiper (O'Reilly and Wingfield 2001). Interestingly, female western sandpipers are known to abandon incubation duties frequently compared with males (referenced in Myers *et al*., 1982), but it is not known whether this abandonment is related to stressors during incubation, or possibly to seek additional mating opportunities as reported in other sandpiper species (Thomas *et al*. 2007). Regardless, the high acute stress response of female White-rumped sandpipers in this study does

not appear to result in increased abandonment since at my site sandpipers and phalaropes are similar in hatch success.

In phalaropes, females had higher baseline and stress-induced CORT than males. Baseline CORT was higher in female phalaropes possibly due to high energetic demands placed on polyandrous females, such as producing multiple clutches in a short period of time (Ross 1979, Liker *et al*. 2001). Baseline levels were similar in courtship and incubating male phalaropes suggesting that the two stages are similar in their energetic requirements, otherwise I might expect the more energy intensive stage to have higher CORT (Landys *et al*. 2006). Using the complete dataset, incubating male phalaropes had the lowest stress-induced CORT levels and the difference between courtship males and females approached significance. However, when I used a subset of the individuals from each stage trapped over a similar time period, females had higher levels than males at either stage. In the smaller dataset, incubating males had lower levels than courtship males but the difference was no longer significant. Therefore, I demonstrate that sex differences in both baseline and stress-induced CORT are present prior to incubation beginning.

I found that in both species, females had higher stress-induced CORT than males, which is contrary to my predictions for sandpipers and contrary to studies of species where sex roles differ (passerines: Astheimer *et al*. 1994, Holberton and Wingfield 2003, Meddle *et al*. 2003). In Pectoral sandpipers (*Calidris melanotus*), a polygynous shorebird where males do not incubate, males had higher stress-induced CORT than females (O'Reilly and Wingfield 2001). Given the close similarities in breeding behaviour between Pectoral and White-rumped sandpipers, it is not clear what aspect of White-rumped sandpiper breeding biology could account for lower stress-induced CORT levels in males.

There have been two important observations suggesting that a dampened stress response may be important for tolerating stressors that could ultimately reduce reproductive success. The most obvious is the observation that the incubating sex has a lower CORT response (O'Reilly and Wingfield 2001). Another is that birds breeding at high latitudes have lower acute CORT, possibly resulting from the need to deal with more extreme weather at northern latitudes (O'Reilly and Wingfield 2001, Silverin *et al*. 1997, Silverin and Wingfield 1998). However, an alternative explanation for these sex differences, which is not mutually exclusive, is that CORT levels may be higher in both sexes in the pre-incubating period or solely in the sex competing for mates as a result of higher reproductive-related stress stemming from shortened breeding seasons at higher latitudes. Indeed, northern species also tend to have higher testosterone levels (Garamszegi *et al*. 2008, Goymann *et al*. 2004). Increased reproductive effort can be accompanied by severe stress-related physiological changes in a variety of animals (e.g., mammals: Boonstra *et al*. 2001; birds: Greives *et al*. 2007), and territorial defence and courtship in birds are themselves energetically costly (Barnett and Briskie 2007, Lynn *et al*. 2010). Because CORT levels are typically higher during the breeding season compared with other stages during the year (Astheimer *et al*. 1994, Romero *et al*. 1997, Romero and Wingfield 1998), higher levels typically found in the competing sex may reflect an adaptive mechanism to deal with stressful and demanding breeding requirements. In phalaropes, the differences in mean values of baseline and stress-induced CORT between females and courtship males, and between courtship males and incubating males suggest that sex differences are due to both conditions facing courtship birds (i.e., polyandrous females), as well as moderate reductions in incubating males. However, the results from sandpipers contradict either mechanism and suggest that these are not general mechanisms.

Predictions 2 and 3: Nest Defence Traits, CORT and Hatch Success

The patterns detected in male phalaropes demonstrate that stress-induced CORT levels are lower among incubating birds. I expect these sex differences to come about via selection for individual phenotypes that facilitate nest defence and prevent nest abandonment. In phalaropes, the strength of nest defence (PC1) was not related to CORT levels (Table 6). However, male phalaropes with lower baseline and stress-induced CORT levels returned more quickly to the nest following a disturbance (PC2). No such relationships were found in sandpipers.

I failed to detect any significant effects of CORT on hatch success. In phalaropes, there were clear tendencies for successful phalaropes to have both lower baseline and stress-induced CORT but the ability to detect a statistically significant effect was hindered by a lack of statistical power. Not only did I have a relatively small sample size, but the sample was also biased towards successful nests. Hatch success for phalaropes was 16% at my site over the years of this study (unpublished data) yet successful nests comprised about 40% of my sample because nests failed faster than they could be trapped. Thus, my sample under-represents failed nests, nests of individuals expected to have the highest CORT levels.

Interestingly, the nest defence variables segregated into two principal components that were similar for both species. The first principal component was comprised of flush distance, secondary distance, and behavioural nest defence while the second component was comprised solely of the time taken to return to the nest following a disturbance. In other words, I found that an individual's tendency to perform a strong nest defence display was not related to nest attendance.

Nest defence behaviours are good candidates to be related to CORT levels because they result in increased energetic expenditure, and involve elements of aggressive confrontation and boldness, all of which are related to CORT and the adrenocortical stress response (Wingfield and

Silverin 1986, Hayden-Hixson and Ferris 1991, DeNardo and Licht 1993, Haller *et al*. 1997, Landys *et al*. 2006, Atwell *et al*. 2012). While nest defence displays (as in PC1 in the present study) improve reproductive success (e.g., Garcia 2003, Goławski and Mitrus 2008), my results suggest that tolerance of the approach of a threat, the intensity of the observed nest defence display and the distance the bird remained from the threat are not related to the adrenocortical axis (Table 6). Consequently, these behaviours could be mediated through hormones other than CORT. For example, prolactin is responsible for eliciting parental behaviours (Buntin 1996) and in particular has been linked to nest attendance and rates of nest desertion (Groscolas *et al*. 2008; Angelier *et al*. 2009a). That CORT and prolactin levels can vary independently of each other (Angelier *et al*. 2009b) suggests that they could have independent effects on breeding behaviour in some species.

A number of studies have shown that artificially elevated levels of CORT or naturally high baseline CORT results in nest abandonment and reduced reproductive success in birds (Silverin 1986, Love *et al*. 2004, Groscolas *et al*. 2008, Angelier *et al*. 2009a, Spée *et al*. 2010, Spée *et al*. 2011). In a number of studies, birds implanted with CORT had hormonal and behavioural effects lasting days, yet in some cases these changes have been interpreted as demonstrating acute changes in CORT (Breuner *et al*. 2008). Acute increases in CORT elicit physiological changes in the span of minutes (Landys *et al*. 2006) and acute changes can have different or even the opposite effects to chronic increases (aggression: see above; energy mobilization/fat deposition: McEwen 2004; immunity: Dhabhar and McEwen 1997, Martin 2009). To my knowledge, there are few studies of the adaptive significance of the natural acute stress response with respect to breeding activity measured at the level of the individual. With that said, my results agree with the studies mentioned above: phalaropes with lower CORT returned

to the nest more quickly following a disturbance and lower stress-induced CORT was related to higher hatch success in both species.

Based on my results, CORT appears to play a role in regulating reproductive-based traits in phalaropes, but not sandpipers. However, I provided only partial support for the idea that sex differences in CORT are related to parental role. Despite both species being exposed to the same environmental conditions (i.e., weather and predation risk), female sandpipers did not conform to my predictions (Table 7). In phalaropes, females generally had higher CORT than males at either the courtship or incubating stage, suggesting that sex differences are not solely due to selection placed upon incubators. I suggest that elevated CORT in the sex competing for mates could play a permissive role in reproductive behaviours while facilitating physiological changes required during this time. However, I found evidence for a decline in CORT from the courtship to incubation stage in male phalaropes, which can be explained by individuals with lower CORT returning to incubate more quickly after a disturbance. In phalaropes, I found a general concordance in how baseline and stress-induced CORT related to my three predictions. More study is needed to understand what environmental or physiological factors account for species differences under similar conditions, because my results suggest that CORT is not regulating the reproductive behaviours of sandpipers.

Literature Cited

Angelier, F., C. Clément-Chastel, J. Weicker, G.W. Gabrielsen, and O. Chastel. 2009a. How does corticosterone affect parental behaviour and reproductive success? A study of prolactin in black-legged kittiwakes. Funct Ecol 23: 784-793.

Angelier, F., M. Børge, S. Blanc, and O. Chastel. 2009b. What factors drive prolactin and corticosterone responses to stress in a long-lived bird species (Snow petrel *Pagodrama nivea*)? Physiol Biochem Zool 82: 590-602.

Astheimer, L.B., W.A. Buttemer and J.C. Wingfield. 1994. Gender and seasonal differences in the adrenocortical response to ACTH challenge in an arctic passerine, *Zonotrichia leucophrys gambelii*. Gen Comp Endocrinol 94: 33-43.

Atwell, J.W., G.C. Cardoso, D.J. Whittaker, S. Campbell-Nelson, K.W. Robertson and E.D. Ketterson. 2012. Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation. Behav Ecol 23: 960-969.

Barnett, C.A. and J.V. Briskie. 2007. Energetic state and the performance of dawn chorus in silvereyes (*Zosterops lateralis*). Behav Ecol Sociobiol 61: 579-587.

Bókony, V., Á.Z. Lendvai, A. Liker, F. Angelier, J.C. Wingfield and O. Chastel. 2009. Stress response and the value of reproduction: Are birds prudent parents? Am Nat 173: 589-598.

Bonier, F., P.R. Martin, I.T. Moore and J.C. Wingfield. 2009. Do baseline glucocorticoids predict fitness? Trends Ecol Evol 24: 634-642.

Boonstra, R., C.J. McColl and T.J. Karels. 2001. Reproduction at all costs: the adaptive stress response of male arctic ground squirrels. Ecology 82: 1930-1946.

Breuner, C.W. and M. Orchinik. 2001. Seasonal regulation of membrane and intracellular receptors in the house sparrow brain. J Neuroendocrinol 13: 412-420.

Breuner, C.W., S.H. Patterson and T.P. Hahn. 2008. In search of relationships between the acute adrenocortical response and fitness. Gen Comp Endocrinol 157: 288-295.

Buntin, J.D. 1996. Neural and hormonal control of parental behaviour in birds. In: Rosenblatt, J.S., Snowdon, C.T, (Ed.), Advances in the Study of Behavior. Academic Press, New York.

Colwell, M.A. 2010. Shorebird Ecology, Conservation, and Management. University of California Press, California.

Cornelius, J.M., C.W. Breuner and T.P. Hahn. 2012. Coping with the extremes: stress physiology varies between winter and summer in breeding opportunists. Biol Lett 8: 312-315.

DeNardo, D.F. and P. Licht. 1993. Effects of corticosterone on social behaviour of male lizards. Horm Behav 27: 184-199.

Dhabhar, F.S. and B.S. McEwen. 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav Immun 11: 286-306.

Edwards, D.B., and H.G. Gilchrist. 2011. A new means of catching shorebirds: the Super Talon net gun. Wader Study Group Bulletin 118: 134-6.

Fridolfsson, A.K. and H. Ellegren. 1999. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 30: 116-121.

Garamszegi, L.Z., K. Hirschenhauser, V. Bókony, M. Eens, S. Hurtez-Boussès, A.P. Møller, R.F. Oliveira and J.C. Wingfield. 2008. Latitudinal distribution, migration, and testosterone levels in birds. Am Nat 172: 533-546.

Garcia, J.T. 2003. Is female investment in defence correlated with breeding success in Hen Harriers *Circus cyaneus*? Bird Study 50: 142-145.

Goławski, A. and C. Mitrus. 2008. What is more important: nest-site concealment or aggressive behaviour? A case study of the red-backed shrike, *Lanius collurio*. Folia Zool Brno 57: 403-410.

Goymann, W., I.T. Moore, A. Scheuerlien, K. Hirschenhauser, A. Grafen and J.C. Wingfield. 2004. Testosterone in tropical birds: Effects of environmental and social factors. Am Nat 164: 327-334.

Greives, T.J., J.M. Casto and E.D. Ketterson. 2007. Relative abundance of males to females affects behaviour, condition and immune function in a captive population of dark-eyed juncos *Junco hyemalis*. J Avian Biol 38: 255-260.

Griffiths, R., M.C. Double, K. Orr and R.J.G. Dawson. 1998. A DNA test to sex most birds. Mol Ecol 7: 1071-1075.

Groscolas, R., A. Lacroix, J.-P. Robin. 2008. Spontaneous egg or chick abandonment in energy-depleted king penguins: a role for corticosterone and prolactin? Horm Behav 53: 51-60.

Haller, J., I. Albert and G.B. Makara. 1997. Acute behavioural effects of corticosterone lack specificity but show marked context-dependency. J Neuroendocrinol 9: 515-518.

Hayden-Hixon, D. M. and C.F. Ferris. 1991. Steroid specific regulation of agonistic responding in the anterior hypothalamus of male hamsters. Physiol Behav 50: 793–799.

Holberton, R.L. and J.C. Wingfield. 2003. Modulating the corticosterone stress response: A mechanism for balancing individual risk and reproductive success in arctic-breeding sparrows? Auk 120: 1140-1150.

Kitaysky, A.S., E.V. Kitaiskaia, J.F. Piatt and J.C. Wingfield. 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. Horm Behav 43: 140-149.

Landys, M.M., M. Ramenofsky and J.C. Wingfield. 2006. Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. Gen Comp Endocrinol 148: 132-149.

Liebezeit, J.R., P.A. Smith, R.B. Lanctot, H. Schekkerman, I. Tulp, S.J. Kendall, D.M. Tracy,

R.J. Rodrigues, H. Meltofte, J.A. Robinson, C. Gratto-Trevor, B.J. McCaffery, J. Morse and S.W. Zack. 2007. Assessing the development of shorebird eggs using the flotation method: Species-specific and generalized regression models. Condor 109: 32-47.

Liker, A., J.D. Reynolds and T. Székely. 2001. The evolution of egg size in socially polyandrous shorebirds. Oikos 95: 3-14.

Love, O.P., C.W. Breuner, F. Vézina and T.D. Williams. 2004. Mediation of a corticosterone-induced reproductive conflict. Horm Behav 46: 59-65.

Lynn, S.E., T.B. Stamplis, W.T. Barrington, N. Weida and C.A. Hudak. 2010. Food, stress, and reproduction: Short-term fasting alters endocrine physiology and reproductive behaviour in the zebra finch. Horm Behav 58: 214-222.

Martin, L.B. 2009. Stress and immunity in wild vertebrates: Timing is everything. Gen Comp Endocrinol 163: 70-76.

McEwen, B.S. 2004. Protection and damage from acute and chronic stress: Allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. Ann N Y Acad Sci 1032: 1-7.

Meddle, S.L., N.T. Owen-Ashley, M.I. Richardson and J.C. Wingfield. 2003. Modulation of the hypothalamic-pituitary-adrenal axis of an Arctic-breeding polygynandrous songbird, the Smith's longspur, *Calcarius pictus*. Proc Biol Sci 270: 1849-1856.

Myers, J.P., O. Hildén and P. Tomkovich. 1982. Exotic *Calidris* species of the Siberian tundra. Ornis Fennica 59: 175-182.

O'Reilly, K.M. and J.C. Wingfield. 2001. Ecological factors underlying the adrenocortical response to capture stress in arctic-breeding shorebirds. Gen Comp Endocrinol 124: 1-11.

O'Reilly, K.M. and J.C. Wingfield. 2003. Seasonal, age, and sex differences in weight, fat reserves, and plasma corticosterone in western sandpipers. Condor 105: 13-26.

R Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Remage-Healey, L. and L.M. Romero. 2001. Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. Am J Physiol Regul Integr Comp Physiol 281: R994– R1003.

Romero, L.M. and J.C. Wingfield. 1998. Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's White-crowned sparrows (*Zonotrichia leucophrys gambelii*). Comp Biochem Physiol 119C: 31-36.

Romero, L.M., M. Ramenofsky and J.C. Wingfield. 1997. Season and migration alters the corticosterone response to capture and handling in an arctic migrant, the White-crowned sparrow (*Zonotrichia leucophrys gambelii*). Comp Biochem Physiol 116C: 171-177.

Ross, H.A. 1979. Multiple clutches and shorebird egg and body-weight. Am Nat 113: 618-622.

Sapolsky, R.M., L.M. Romero and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21: 55-89.

Silverin, B. 1986. Corticosterone-binding proteins and behavioural effects of high plasma levels of corticosterone during the breeding period. Gen Comp Endocrinol 64: 67-74.

Silverin, B. and J.C. Wingfield. 1998. Adrenocortical responses to stress in breeding pied flycatchers, *Ficedula hypoleuca*: relation to latitude, sex and mating status. J Avian Biol 29: 228-234.

Silverin, B., B. Arvidsson and J.C. Wingfield. 1997. The adrenocortical responses to stress in breeding willow warblers, *Phylloscopus trochilus*, in Sweden: effects of latitude and gender. Funct Ecol 11: 376-384.

Smith, P.A. and S. Wilson. 2010. Intraseasonal patterns in shorebird nest survival are related to nest age and defence behaviour. Oecologia 163: 613-624.

Spée, M., M. Beaulieu, A. Dervaux, O. Chastel, Y. Le Maho and T. Raclot. 2010. Should I stay or should I go? Hormonal control of nest abandonment in a long-lived bird, the Adélie penguin. Horm Behav 58: 762-768.

Spée, M., L. Marchal, D. Lazin, Y. Le Maho, O. Chastel, M. Beaulieu and T. Raclot. 2011. Exogenous corticosterone and nest abandonment: a study in a long-lived bird, the Adélie penguin. Horm Behav 60: 362-370.

Thomas, G.H., T. Székely and J.D. Reynolds. 2007. Sexual conflict and the evolution of breeding systems in shorebirds. Adv Study Behav 37: 279-342.

Walsh, C. and R. Mac Nally. 2013. hier.part: Hierarchical Partitioning. R package version 1.0-4. http://CRAN.R-project.org/package=hier.part

Washburn, B.E., D.L. Morris, J.J. Millspaugh, J. Faaborg and J.H Schulz. 2002. Using a commercially available radioimmunoassay to quantify corticosterone in avian plasma. Condor 104: 558–563.

Wingfield, J.C. and B. Silverin. 1986. Effects of corticosterone on territorial behaviour of free-living male song sparrows, *Melospiza melodia*. Horm Behav 20: 405-417.

Wingfield, J.C., H. Schwabl and P.W. Mattocks Jr. 1990. Endocrine mechanisms of migration. In: Gwinner, E. (Ed.), Bird Migration: Physiology and Ecophysiology. Springer-Verlag, New York.

Wingfield, J.C., C.M. Vleck and M.C. Moore. 1992. Seasonal changes in the adrenocortical response to stress in birds of the Sonoran Desert. J Exp Zool 264: 419-428.

Wingfield, J.C., D.L. Maney, C.W. Breuner, J.D. Jacobs, S. Lynn, M. Ramenofsky and R.D. Richardson. 1998. Ecological bases of hormone-behavior interactions: the "emergency life history stage". Am Zool 38: 191-206.

CHAPTER 4: Sex-biased immunocompetence in a sex-role reversed bird.

Abstract

Across taxa, males tend to have relatively poor immune function compared with females. This pattern is thought to result from reproductive-based trade-offs: in males, high investment in current reproduction results in reduced investment in traits conferring a survival advantage, such as immune function. Testosterone (T) is thought to mediate these trade-offs in males, acting directly on the immune system or indirectly either through T-mediated behaviours or other hormones such as corticosterone (CORT). I used a sex-role reversed bird, the Red phalarope (*Phalaropus fulicarius*), to provide a unique approach to understanding the mechanisms underlying sex-biased immunocompetence. Sex-role reversed systems afford a unique opportunity to uncouple the relationships among sex, high reproductive potential, and general hormone levels that are found in typical systems. As predicted, females had lower levels of immunoglogulins and lymphocytes, but there were no sex differences in haptoglobin or heterphils. Because I also found that females had low levels of T and high levels of CORT, sex differences in immunity appear to be consistent with CORT-mediated immunosuppression; high levels of T in males did not result in reduced immunocompetence in males. However, within sex analyses showed females with high T had low immunoglobulin levels (but higher lymphocyte levels). T was not related to immunity in males, but males with higher CORT had lower lymphocyte levels. These results suggest that T and CORT mediate immunity in a sex-specific fashion that is not dose-dependent. The findings of my study support the notion of the sex with the highest reproductive potential – females of the sex-roled reversed Red phalarope – reducing investment in traits that confer a survival advantage.

Introduction

Bateman's principle (Bateman 1948, Trivers 1972) posits that females maximize their reproductive potential by investing in longevity, whereas males improve their reproductive potential through high mating rates. Bateman's principle, and the different male and female trajectories that it predicts, is thought to be the primary explanation for sex differences in reproductive investment (Bateman 1948, Trivers 1972, Clutton-Brock 1988, Jones 2009). Given finite resources, I therefore expect males to invest more resources in behavioural and physical traits that confer proximate fitness advantages at the expense of those conferring a survival advantage.

Immune function is an important life history trait that is subject to trade-offs with other necessary activities, such as reproduction (Sheldon and Verhulst 1996, Rauw 2012). Males seeking to achieve high levels of reproductive success may do so at the expense of investment in immune function (Rolff 2002). On the other hand, females should invest more into immunity than males because females improve reproductive success by increasing their reproductive lifespan (Rolff 2002). Consistent with this prediction, across a broad range of taxa, female immune function is often superior to that of males (Zuk and McKean 1996, Klein 2000, Nunn *et al*. 2009).

In vertebrates, testosterone (T) is thought to be an important regulator of the degree of investment in reproduction (Hau 2007, Evans 2010, Hau *et al*. 2010). In males, T initiates spermatogenesis, triggers breeding behaviours such as courtship and territoriality, and promotes the expression of secondary sexual traits (Gill 2007). T also is widely considered immunosuppressive, and as a result, sex differences in immune function are often attributed to T expression. This immunosuppressive role of T is an important component of sexual selection

theory because it is thought to maintain honesty in the expression of T-mediated traits through the immunocompetence handicap hypothesis (ICHH: Folstad and Karter 1992).

However, the immunosuppressive role of T is debated (e.g., Roberts *et al*. 2004), and several studies suggest revisions to the hormonal mechanisms behind T-mediated immunosuppression (Owen-Ashley *et al*. 2004, Fuxjager *et al*. 2011). Much of the attention has been on glucocorticoids as providing the immunosuppressive effect attributed to T, resulting in, for example, a stress-mediated version of the ICHH (Møller 1995, Roberts *et al*. 2007). Similar to T, corticosterone (CORT) may mediate reproductive-based trade-offs (Hau *et al*. 2010) by allocating resources away from other functions, such as immunocompetence. In fact, the evidence for an immunosuppressive role for CORT is more substantial than for T (Råberg *et al*. 1998, Buchanan 2000, Sapolsky *et al*. 2000).

Differentiating between the original and stress-mediated versions of the ICHH is tricky given the inter-relationships between T and CORT. Like T, CORT is elevated during the breeding season compared with other times of the year (Silverin and Wingfield 1982, Romero and Wingfield 1998, Breuner and Orchinik 2001), and artificially increasing T results in an increase in CORT (Ketterson *et al*. 1991, Klukowski *et al*. 1997, Schoech *et al*. 1999, Evans *et al*. 2000, Poiani *et al*. 2000, Casto *et al*. 2001, Mateos 2005, Ashley *et al*. 2009). Furthermore, T may also influence CORT by affecting the production and binding ability of corticosterone-binding globulin (CBG: Klukowski *et al*. 1997, Breuner and Orchinik 2001, Swett and Breuner 2008). As a result, the immunosuppressive action ascribed to T may operate directly or indirectly through CORT (Møller 1995, Owen-Ashley *et al*. 2004). Because of the relationship between T and CORT, the challenge to understanding the hormonal mechanism resulting in breeding-related immunosuppression involves separating the effects of T from those of CORT. To date,

attempts to do this have found disparate results. Evans *et al*. (2000) reported an immunoenhancing role for T and a suppressive effect for CORT. In contrast, Roberts *et al*. (2007) found no immunomodulating effect of T and CORT separately, but rather that high levels of both hormones interacted to stimulate the antibody response. In subsequent work, Roberts *et al*. (2009) once again found no immunomodulating effect of T when T levels were manipulated in quail (*Coturnix japonica*) bred for low and high CORT responsiveness. However, in this study both high and low CORT quail lines had lower humoral immunity than quail with natural CORT responsiveness.

Critical tests of the mechanisms underlying sex differences in immunity, including the hormonal mechanisms that mediate sex-biased immunocompetence (SBIC), are rare because cases in which the influence of sex can be separated from other factors are exceptional in vertebrates. Generalizations about SBIC are predicated on the typical roles of males and females, and so a novel approach to studying these life history trade-offs lies in using species with nonconventional sex roles (see Roth *et al*. 2011). Such systems are useful for testing hypotheses of sexual selection (Williams 1975), and in particular, sex differences in immunity (Forbes 2007). My aim in this study was to test for sex-differences in immunity in a sex-role reversed (SRR) shorebird, the Red phalarope (*Phalaropus fulicarius*), as well as to address the hormonal mechanisms that may be responsible for SBIC.

SRR species provide a natural experiment of sorts in which typical reproductive roles have been uncoupled from sex. In birds exhibiting sex-role reversal, males usually have higher T than females (Fivizzani and Oring 1986, Fivizzani *et al*. 1986, Schlinger *et al*. 1989, Goymann and Wingfield 2004, Muck and Goymann 2010; but see Höhn and Cheng 1967, Höhn 1970 who found higher gonadal T levels in females). In terms of CORT, previous work on Red phalaropes

has shown that females have higher levels of CORT than males (O`Reilly and Wingfield 2001, Edwards *et al*. 2013: Chapter 3), a pattern that differs from many species with female-biased parental care where females have lower levels of CORT (Astheimer *et al*. 1994, O`Reilly and Wingfield 2001, Holberton and Wingfield 2003, Meddle *et al*. 2003). So, unlike traditional systems in which males tend to have higher breeding levels of both T and CORT, phalaropes may provide a unique opportunity to independently examine the influence of these hormones on SBIC in a natural system.

The main goal of this study was to test for the presence/directionality of sex-biased immunocompetence in a role-reversed shorebird, the Red phalarope, while examining the hormonal mechanisms behind SBIC to determine whether SBIC was consistent with a Bateman's-like trade-off, or with a T-mediated handicap, as in the ICHH. Polyandrous female phalaropes have higher reproductive potential than males, and express traits, such as showy plumage and courtship behaviours (Tracy *et al*. 2002), that are more typically seen in males facing high levels of competition for mates. I predicted reduced immune function in females resulting from a Bateman's-like trade-off (Rolff 2002), coincident with higher levels of baseline CORT in females (Chapter 3). Given previous studies of SRR species, I expected high levels of T in males, but I did not predict high T in males to result in male-biased immunosuppression, as would be expected if T functions in an obligatory, dose-dependent manner. Finally, if variation in immune function is related to CORT rather than T, I predicted that within a sex, individuals with higher CORT, but not higher T, would have reduced immune function.

Methods

I trapped Red phalaropes (*Phalaropus fulicarius*) at East Bay Migratory Bird Sanctuary, Nunavut, Canada (N63°59'13.5" W81°41'48.3") during reproductive activities in 2009 and 2010. Phalaropes tend to arrive in late May-early June and form aggregations of up to 20 birds in early melt ponds. Nesting typically commences by mid-June for early nesting birds but newly laid clutches can be found as late as mid-July at this site (personal observation). I captured males and females during the courtship, pre-egg laying period when males and females perform relatively similar behaviours compared with other times during the breeding season; neither sex is defensive of a territory (Tracy *et al*. 2002). Capture effort was focussed on birds early in the season in order to reduce the likelihood of including females that had laid previous clutches, males that had lost clutches and re-entered the breeding population, or individuals of either sex that were no longer reproductively active. Males (mean trap date 172 Julian Date; range 160-187) and females (mean trap date 170 Julian Date; range 161-187) were trapped during similar time periods. I ensured all females used were prelaying birds by inspecting the cloaca; I excluded two females from the analysis because they were carrying unlaid eggs.

Trapping and Blood Sampling

I trapped phalaropes using a Super Talon Net Gun (Fly Dragon Technology Co., Ltd) as reported in Edwards and Gilchrist (2011). To ensure my samples reflected baseline plasma hormone levels, I started a stopwatch as soon as the trap was triggered and collected a blood sample from the ulnar vein within 3-4min. Most blood samples (>90%) were taken in less than 3min from the time of capture. Samples were kept cool until they could be centrifuged (<6hrs). Plasma was initially frozen at -20°C in the field, and later transferred to -80°C until assaying in the lab.

Immunological Measures

I assessed immunocompetence using three measures of immunity. Immunoglobulin Y (IgY) is the main class of antibody involved in the adaptive immune response in birds (Roitt *et al*. 1993, Lundqvist *et al*. 2006). I used an enzyme-linked immunosorbent assay (ELISA) technique based on Martínez *et al*. (2003) and Bourgeon *et al*. (2006) to measure IgY in the plasma using a commercial anti-chicken IgY antibody (Sigma-Aldrich: A-9046). This assay has been validated in five species of birds representing diverse lineages (Martínez *et al*. 2003). I optimized the assay for phalaropes using serial dilutions of plasma. The optimal concentration (1:4000) lays at the centre of the region of linear change in the sigmoidal relationship between absorbance and plasma IgY concentration. Microplates were read at 405nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA; used for all assasy in this study). Intra-assay variation was 2.7% based on duplicate samples and inter-plate assay variation was 4.9% based on chicken IgY control.

I made a blood smear in the field at the time of blood collection to assess leukocyte numbers. Smears were stained with Protocol Hema 3 (Fisher Scientific, Toronto, CAN) and leukocytes were counted per 10,000 red blood cells for relative leukocyte density. Although there are five leukocyte types which I tallied to give total counts, I restricted the statistical analyses to the most common cell types: heterophils and lymphocytes. Heterophils are phagocytic cells involved in innate responses, but also function in cell-mediated immunity and humoral immunity; lymphocytes are responsible for antibody production (Roitt *et al*. 1993). Slides were also scanned for blood parasites; however, none were found. Blood smears were counted for samples collected in 2009 only.

As an index of innate immune function, I quantified levels of haptoglobin. Haptoglobin and related haptoglobin-like proteins are involved with the acute phase response (Owen-Ashley

and Wingfield 2007). The acute phase response is activated early in infection and acute phase proteins can improve opsonisation of antigens and phagocyte function, as well as activating the complement cascade (Owen-Ashley and Wingfield 2007). Haptoglobin in particular binds to resources necessary for growth and replication of bacteria (Quaye 2008) and, in mammals, plays a broad role in regulating lymphocyte tissue including lymphocyte activity (Huntoon *et al*. 2008) and activates innate immune responses (Shen *et al*. 2012). To measure plasma haptoglobin levels, I used a commercially available kit (Tri-Delta Diagnostics, Morris Plains, NJ, USA) that has been used recently to determine levels of haptoglobin in other species of Charadriiformes (Buehler *et al*. 2010, Berzins *et al*. 2011). I ran samples in triplicate following the manufacturer's instructions and read the absorbance at 630nm. Hemolyzed samples were not used in the assay. The intra-assay variation was calculated from triplicate samples at 2.1% and the inter-plate assay variation was 5.9% based on controls provided with the kit.

Hormonal Assays

I determined plasma T levels (pg/mL) for a subset of individuals for which I had sufficient plasma. I used a commercially available ELISA kit (Salimetrics, State College, Pennsylvania PA, USA) that has been shown to effectively measure T levels in birds (Washburn *et al*. 2007). I validated the kit for this species by confirming parallelism. The intra-assay variation based on duplicates was 3.8% and the inter-plate assay variation for this assay was 11.1%.

These CORT measures are part of a larger study and my methodology for measuring CORT is outlined in greater detail in previous work (Chapter 3: Edwards *et al*. 2013). Although the CORT values have been reported previously (Chapter 3: Edwards *et al*. 2013), they have not been analyzed previously in the context of variation in T or indices of immune function. The

intra-assay variation for duplicate CORT samples was 7.1% and the inter-plate assay variation was 14.0%.

Statistical Analyses

I trapped 38 females and 40 males. I was prevented from doing all assays for every individual because of small sample volume, resulting in different sample sizes among physiological measures.

I tested for differences between males and females in physiological measures (dependent variables) using linear models incorporating year and date as factors. I used mass adjusted for body size as a measure of condition by using mass (g) as the dependent variable and including tarsus length (mm) as a factor. Data were approximately normally distributed except for CORT and T. In the case of T, the distribution was noticeably bimodal, and I tested this presumption using a Dip test (Hartigan and Hartigan 1985). To explore this bimodality, I used logistic regression to determine whether belonging to either the high mode or low mode was related to trap date, whether the male was observed to be paired with a mate at the time of capture and the elapsed time to first bleed, while controlling for year. CORT and T values were square-root and rank transformed respectively.

To determine whether T or CORT predicted immune measures within each sex, I also used linear models with date and year as factors. I previously determined that the elapsed time from being trapped until the blood was taken influenced CORT values (Edwards *et al*. 2013), and so I included elapsed time as a covariate in analyses involving CORT. Because male T values were bimodal, intra-sexual analyses involving male T were done two ways. First, I used the rank transformed data. Additionally, I standardized T values within each T mode and then ran a linear model analysis to determine whether having high T values within a group predicted

immune measures. This may be the case if the high and low mode individuals were in a different state of preparedness for breeding, but in doing so I make the assumption that within each mode the relationship between T and immune measures is similar. However, the interpretation of the results did not differ between these two approaches and so I report only the results using the rank transformed T values. All statistical analyses were performed in R (R Core Team 2012) as well as the 'diptest' package (Maechler 2012).

Results

Sex Differences in Immunological Measures

As predicted, males had significantly higher IgY levels than females (Sex: $t_{1,75}$=2.37, P=0.02; Year: $t_{1,75}$=0.75, P=0.46; Trap Date: $t_{1,75}$=4.52, P<0.001; Fig. 4A). The trend was similar for lymphocytes (Sex: $t_{1,52}$=2.00, P=0.05; Trap Date: $t_{1,52}$=5.98, P<0.001; Fig. 4B) but there was no difference between the sexes in heterophils (Sex: $t_{1,52}$=0.64, P=0.95; Trap Date: $t_{1,52}$=0.47, P=0.64) or haptoglobin (Sex: $t_{1,58}$=-1.10, P=0.28; Year: $t_{1,58}$=-0.77, P=0.44; Trap Date: $t_{1,58}$=-2.24, P=0.03).

Sex Differences in Hormone Levels and Body Condition

T levels were higher in males (Sex: $t_{1,26}$=2.65, P=0.01; Year: $t_{1,26}$=0.56, P=0.58; Trap Date: $t_{1,26}$=-1.82, P=0.08; Fig. 5A). Male T levels were bimodally distributed (dip statistic, D=0.12, P=0.03), with one group of very high T titres (>950 pg/mL, n=8) and another with low values (<500 pg/mL, n=7). The low cluster was within the bounds of T values of females and the high cluster in males was higher than all female T values. The clusters were not obviously explained by whether the birds were paired to a mate (logistic regression: $Z_{4,15}$=0.32, P=0.75), trap date within the season ($Z_{4,15}$=1.33, P=0.19) or by the amount of time elapsed until the blood sample was taken ($Z_{4,15}$=1.32, P=0.19). Although there was a very weak tendency for individuals trapped later in the season to have lower levels of T, members of the low T cluster were trapped

throughout the courtship stage; no immunological traits measured in this study were bimodal. Females had higher mass corrected for size, an index of condition, than males (Sex: $t_{1,74}$= 6.25, P<0.001; Year: $t_{1,74}$=4.91, P<0.001; Trap Date: $t_{1,74}$=2.73, P=0.008; Tarsus: $t_{1,74}$=2.03, P=0.05; Fig. 5B).

Intra-sexual Variation in Hormones and Immune Measures

Females with high T levels had lower levels of IgY (P=0.03, Table 8), yet higher numbers of lymphocytes (P=0.01). Neither heterophils nor haptoglobin was related to T, and none of the immune measures was related to CORT in females (Table 8).

In males, the bimodality of T levels proved problematic during the analyses, but I found no evidence to support a relationship between immune measures and T (Table 8). As predicted, males with higher levels of CORT had fewer lymphocytes (P=0.02, Table 8).

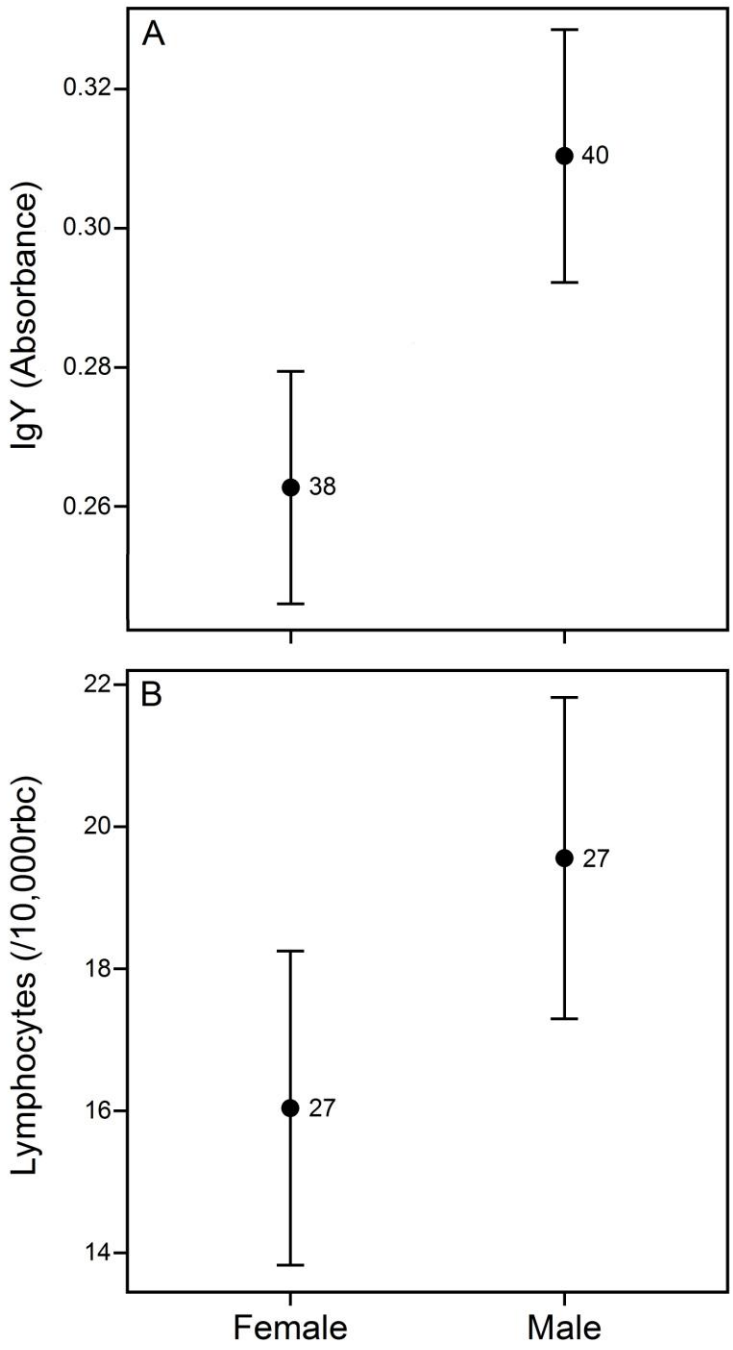


Fig. 4. Immunoglobulin (IgY) concentration (A; P=0.02) and lymphocyte counts (B; P=0.05) were lower in female Red phalaropes trapped during the courtship period. Bars represent standard error.

Table 8. Relationships between immune measures and hormone levels for males and females. Bleed time, the elapsed time between capture and the completion of the first bleed, was included as a factor in analyses involving corticosterone. See text for a description of the treatment of male testosterone levels.

		Testosterone			Corticosterone		
Females		n	t	P	n	t	P
IgY	Hormone	14	-2.53	**0.03**	38	0.68	0.5
	Date		2.14	0.06		4.15	**0.0002**
	Year		-0.85	0.41		0.12	0.91
	Bleed Time					-1.93	0.06
Lymphocytes	Hormone	12	3.06	**0.01**	29	-0.74	0.47
	Date		3.74	**0.005**		3.71	**0.001**
	Bleed Time					-1.01	0.32
Heterophils	Hormone	12	0.55	0.60	29	-0.06	0.95
	Date		-0.92	0.38		0.28	0.79
	Bleed Time					-0.37	0.71
Haptoglobin	Hormone	11	1.14	0.29	27	0.88	0.39
	Date		-0.75	0.48		-1.93	0.07
	Year		1.75	0.12		-0.78	0.44
	Bleed Time					1.11	0.28
Males		n	t	P	n	t	P
IgY	Hormone	15	0.22	0.83	39	0.72	0.47
	Date		2.06	**0.05**		2.55	**0.02**
	Year		0.63	0.53		1.53	0.13
	Bleed Time					-0.67	0.51
Lymphocytes	Hormone	13	0.69	0.50	27	-2.47	**0.02**
	Date		3.89	**0.0007**		3.31	**0.003**
	Bleed Time					2.51	**0.02**
Heterophils	Hormone	13	0.34	0.74	27	-0.58	0.57
	Date		0.35	0.73		0.13	0.90
	Bleed Time					0.77	0.45
Haptoglobin	Hormone	15	1.55	0.13	34	-0.8	0.43
	Date		-0.27	0.79		-0.57	0.57
	Year		-0.11	0.92		0.48	0.63
	Bleed Time					-0.28	0.78

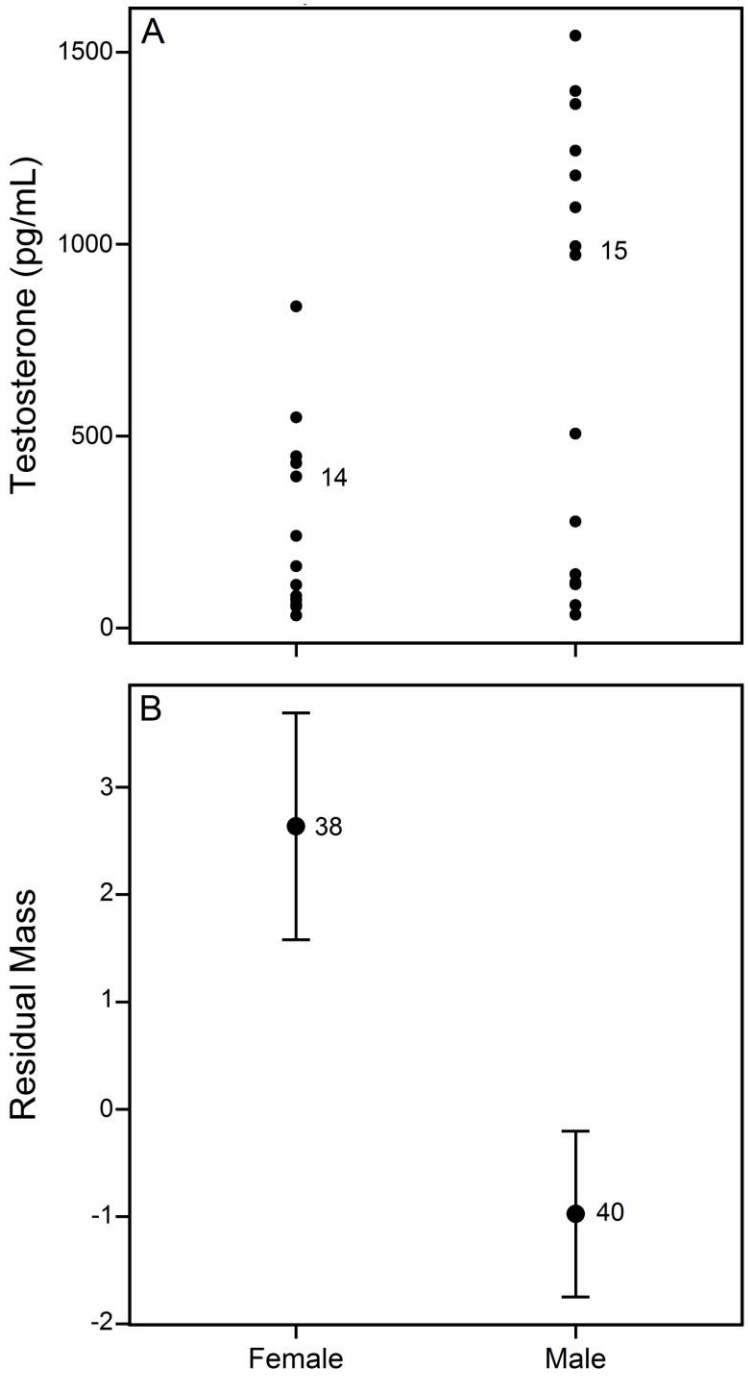


Fig. 5. Male phalaropes had higher testosterone (A; P=0.01) and lower body condition (B; P=0.001) than females. Male testosterone titres were plotted so as to show the bimodal distribution and the analysis was performed on rank transformed data. I interpreted body condition as the partial effect of mass accounting for body size (tarsus), however I used the residual values from the regression of body mass on body size in the plot. Bars represent standard error.

Discussion

Investment in high reproductive rates by males of traditional systems is thought to reduce investment in other traits (e.g., Bateman 1948, Sheldon and Verhulst 1996), which may result in the generally observed pattern of lower immunocompetence in males (Zuk and McKean 1996, Klein 2000, Nunn *et al*. 2009). Until recently, studies of sex-specific reproductive investment and immune function have been limited to species with conventional sex roles, despite the potential for systems demonstrating atypical behaviours to contribute greatly to our understanding of the underlying processes. In contrast to species with conventional sex roles, I found that females of the SRR Red phalarope had lower immunocompetence than males, which supports the idea that life history constraints drive sex differences in immunity (Rolff 2002, Nunn *et al*. 2009). In phalaropes, females have the most to gain from investment in current reproduction, and do so at the expense of traits that confer advantages for future reproduction and longevity (e.g., immune function). That Roth *et al*. (2011) recently reported a similar finding in the SRR pipefish (*Syngnathus typhle*), suggests there may be a general pattern across taxa.

Among the indices of immune function, I saw sex differences in the lymphoid measures (i.e. IgY concentration and lymphocytes), but not innate factors (i.e. haptoglobin and heterophils), which is possibly related to sex differences in energetic requirements. Across species, there is evidence that innate immune measures may be preferentially maintained in the face of energetic challenge and stress (Davis *et al*. 2008). For example, during the energetically costly incubation phase (Gabrielsen *et al*. 1991), female eider ducks (*Somateria mollissima*) maintain levels of innate immunity (Bourgeon *et al*. 2007) despite reductions in humoral measures (Hanssen *et al*. 2005), including IgY levels (Bourgeon *et al*. 2006). Similarly, although mallards (*Anas platyrhynchos*) on restricted diets faced suppression of both humoral and innate immunity, innate immunity recovered shortly after fuel reserves returned to normal levels

whereas antibody levels did not (Bourgeon *et al*. 2010). High levels of antibodies during the reproductive period may be particularly costly for females of polyandrous species; domestic birds bred for high antibody production take longer to reach sexual maturity, produce fewer eggs and generally have reduced lifetime fertility (Siegel *et al*. 1982; Martin *et al*. 1990). Since the production of multiple clutches is at least theoretically important for polyandrous birds, it may therefore be prudent for female phalaropes to down-regulate some aspects of immune function in the face of an energetic challenge, yet maintain other aspects of immune function.

T-mediated immunosuppression is a fundamental explanation for SBIC (Folstad and Karter 1992); however, subsequent research has proposed a role for CORT (Møller 1995; Roberts *et al*. 2007). If T functions in an obligatory, dose-dependent manner, I would expect that males would be immunosuppressed relative to females by virtue of male phalaropes having higher T. However, despite males having higher circulating T, they also had higher values for some immune measures suggesting that high levels of T are not universally immunosuppressive. Although sex differences in immune measures are superficially supported by the notion of CORT-mediated immunosuppression, the relationships between hormones and immune measures within each sex supported a different set of conclusions than those between sexes.

Within sex analyses suggested that T contributes to the regulation of immune function in females but not males, and that CORT modulates immune function in males but not females. In females, IgY was negatively correlated with levels of T, while lymphocyte numbers were positively related to T. Although the direction was opposite, it is worth noting that T was correlated to IgY and lymphocytes, the two measures that showed differences between the sexes. With that said, T-mediated immunosuppression can only explain IgY levels, because individuals with higher T had more lymphocytes. In phalaropes, T may be interacting directly with

lymphocytes to limit the production of immunoglobulins as it does in other species (Kanda *et al*. 1996; see also Hillgarth *et al*. 1997), rather than reducing the production of lymphocytes themselves. However, androgen receptors have not been identified on avian leukocytes to my knowledge, although they do occur on leukocytes in other vertebrates (Slater *et al*. 1995, Liva and Voskuhl 2001). Within males, I found a large range of T levels conforming to a group of high T males and a group with low T (approximately a 7-fold difference in mean T levels between the groups). If T was influencing immunity in males, I would expect to observe bimodality in immune measures but that was not the case. Nor did I find any relationship within males between T and immune measures using different statistical approaches. However, males with higher levels of CORT had fewer lymphocytes supporting an immunosuppressive effect of CORT in males; CORT was not related to any immune measures in females.

There has been discussion as to whether T-mediated life history trade-offs evolve as a unit, with species level variation being the result of differences in dosage of circulating T (evolutionary constraint hypothesis), or whether T-mediated traits and signaling mechanisms can evolve independently (evolutionary potential hypothesis: see Hau 2007). In my study, there was no dose dependent T-mediated effect, as would be expected in the evolutionary constraint hypothesis, because high levels of T did not affect immune measures in males but did in females. Thus, the functional flexibility of T in this system is likely achieved through differences in receptor affinity and/or density, or through metabolism of T to other biologically active forms (Ball and Balthazart 2008). For example, in SRR coucals (*Centropus grillii*) agonistic and courtship behaviours resulted not from higher androgen levels in females, but rather were due to increased sensitivity to androgens resulting from greater receptor expression in the brain (Voigt and Goymann 2007). Similarly in Wilson's phalaropes (*Phalaropus tricolor*), females had higher

5α- and 5β-reductase activity, and as a consequence, may have high rates of T conversion to other biologically active forms in target tissues (Schlinger *et al*. 1989). In addition to the metabolic derivatives produced by reductase activity, Owen-Ashley *et al*. (2004) suggested that T could influence other hormones, such as CORT, which subsequently suppress the immune system. Although the CORT-induced immunosuppression is well documented (Råberg *et al*. 1998, Buchanan 2000, Sapolsky *et al*. 2000), and female phalaropes have higher CORT than males (Edwards *et al*. 2013) yet fewer immunoglobulins and lymphocytes, I found no evidence in the current study that within females, immune measures were suppressed due to CORT.

How the maternal phenotype affects offspring is currently of great interest (Mousseau *et al*. 2009). Specifically, two avenues have received attention: 1) maternal transfer of immunoglobulins, and 2) the influence of maternal CORT. IgY transferred by the female to the developing eggs is important because it serves to bolster the offspring's immunity prior to the development of the chick's own acquired immune system (Grindstaff *et al*. 2006, Grindstaff 2008). Similarly, high levels of maternal CORT can have a variety of effects on offspring such as reduced growth rate and immunity, and skewed sex ratios (Hayward and Wingfield 2004, Love *et al*. 2005, Pryke *et al*. 2014). Female phalaropes, in relation to males, experience both reduced plasma IgY (the present study) and elevated CORT (Chapter 3: Edwards *et al*. 2013) during the period when egg formation occurs, which could have negative consequences for offspring quality. Further studies in both of these areas would be informative but preliminary work suggests that, despite low levels of IgY in females compared with males, this sex-biased immunosuppression does not impair the ability of females to provision eggs with IgY compared with other species of shorebirds (Edwards *et al.* 2014).

I found that indices of immune function in females of the polyandrous Red phalarope are lower than in males. Thus, my findings support the notion that characteristics of the Bateman principle explain investment in immune function. Using this system, I also demonstrate that the roles T and CORT play in mediating reproduction-immune function trade-offs are complicated. My results demonstrate that mere sex differences in the levels of immunomodulating hormone are insufficient in themselves to explain sex differences in immunity. Despite higher levels of CORT and lower levels of T, females were immunocompromised relative to males. Furthermore, I found disparities in how hormones may mediate these differences between the sexes; chiefly, T appears to be immunomodulating in females but not males. Further studies into this system should use an experimental approach to investigate how T interacts with immune components to regulate immune function differently in each sex.

Literature Cited

Ashley, N.T., Q.R. Hays, G.E. Bentley and J.C. Wingfield. 2009. Testosterone treatment diminishes sickness behavior in male songbirds. Horm Behav 56: 169-176.

Astheimer, L.B., W.A. Buttemer and J.C. Wingfield. 1994. Gender and seasonal differences in the adrenocortical response to ACTH challenge in an arctic passerine, *Zonotrichia leucophrys gambelii*. Gen Comp Endocrinol 94: 33-43.

Ball, G.F. and J. Balthazart. 2008. Individual variation and the endocrine regulation of behaviour and physiology in birds: a cellular/molecular perspective. Philos Trans R Soc Lond Biol Sci 363: 1699-1710.

Bateman, A.J. 1948. Intra-sexual selection in *Drosophila*. Heredity 2: 349-368.

Berzins, L.L., H.G. Gilchrist, K.D. Matson and G. Burness. 2011. Sex-specific effects of increased incubation demand on innate immunity in black guillemots. Physiol Biochem Zool 84: 222-229.

Bourgeon, S., F. Criscuolo, Y. Le Maho and T. Raclot. 2006. Phytohemagglutinin response and immunoglobulin index decrease during incubation fasting in female common eiders. Physiol Biochem Zool 79: 793-800.

Bourgeon, S., T. Raclot, Y. Le Maho, D. Ricquier and F. Criscuolo. 2007. Innate immunity, assessed by plasma NO measurements, is not suppressed during the incubation fast in eiders. Dev Comp Immunol 31: 720-728.

Bourgeon, S., M. Kauffmann, S. Geiger, T. Raclot and J.P. Robin. 2010. Relationships between metabolic status, corticosterone secretion and maintenance of innate and adaptive humoral immunities in fasted re-fed mallards. J Exp Biol 213: 3810-3818.

Breuner, C.W. and M. Orchinik. 2001. Seasonal regulation of membrane and intracellular receptors in the house sparrow brain. J Neuroendocrinol 13: 412-420.

Buchanan, K.L. 2000. Stress and the evolution of condition-dependent signals. Trends Ecol Evol 15: 157-160.

Buehler, D.M., B.I. Tieleman and T. Piersma. 2010. Indices of immune function are lower in Red knots (*Calidris canutus*) recovering protein than in those storing fat during stopover in Delaware Bay. Auk 127: 394-401.

Casto, J.M., V. Nolan Jr and E.D. Ketterson. 2001. Steroid hormones and immune function: Experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). Am Nat 157: 408-420.

Clutton-Brock, T.H. 1988. Reproductive Success. University of Chicago Press, Chicago.

Edwards, D.B. and H.G. Gilchrist. 2011. A new means of catching shorebirds: the Super Talon net gun. Wader Study Group Bulletin 118: 134-6.

Edwards, D.B., E.H. Chin, G. Burness, A.I. Schulte-Hostedde and H.G. Gilchrist. 2013. Linking sex differences in corticosterone with individual reproductive behaviour and hatch success in two species of uniparental shorebirds. Comp Biochem Physiol A 166: 169-176.

Edwards, D.B., M. Haring, H.G. Gilchrist and A.I. Schulte-Hostedde. 2014. Do social mating systems limit maternal immune investment in shorebirds? Can J Zool 92: 223-228.

Evans, M.R. 2010. Why does testosterone influence morphology, behaviour and physiology? The Open Ornithology Journal 3: 21-26.

Evans, M.R., A.R. Goldsmith and S.R.A. Norris. 2000. The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 47: 155-163.

Fivizzani, A.J. and L.W. Oring. 1986. Plasma steroid hormones in relations to behavioral sex role reversal in the Spotted Sandpiper, *Actitis macularia*. Biol Reprod 35: 1195-1201.

Fivizzani, A.J., M.A. Colwell and L.W. Oring. 1986. Plasma steroid hormone levels in free-living Wilson's Phalaropes, *Phalaropus tricolor*. Gen Comp Endocrinol 62: 137-144.

Folstad, I. and A.J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603-622.

Forbes, M.R. 2007. On sex differences in optimal immunity. Trends Ecol Evol 22: 111-113.

Fuxjager, M.J., J. Foufopoulos, R. Diaz-Uriarte and C.A. Marler. 2011. Functionally opposing effects of testosterone on two different types of parasite: implications for the immunocompetence handicap hypothesis. Funct Ecol 25: 132-138.

Gabrielsen, G.W., F. Melhum, H.E. Karlsen, Ø. Andresen and H. Parker. 1991. Energy cost during incubation and thermoregulation in the female Common Eider *Somateria mollissima*. – Norsk Polarinstitutt Skrifter 195: 51–62.

Gill, F.B. 2007. Ornithology 3rd ed. W.H. Freeman and Company, New York.

Goymann, W. and J.C. Wingfield. 2004. Competing females and caring males. Sex steroids in African Black Coucals, *Centropus grillii*. Anim Behav 68: 733-740.

Grindstaff, J.L. 2008. Maternal antibodies reduce costs of an immune response during development. J Exp Biol 211: 654-660.

Grindstaff, J.L., D. Hasselquist, J-Å Nilsson, M. Sandell, H.G. Smith and M. Stjernman. 2006. Transgenerational priming of immunity: maternal exposure to a bacterial antigen enhances offspring humoral immunity. Proc Biol Sci 273: 2551-2557.

Hanssen, S.A., D. Hasselquist, I. Folstad and K.E. Erikstad. 2005. Cost of incubation in a long-lived bird: incubation effort reduces immune function and future reproduction. Proc Biol Sci 272: 1039-1046.

Hartigan, J.A. and P.M. Hartigan. 1985. The Dip Test of Unimodality. Ann Stat 13: 70–84.

Hau, M. 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. BioEssays 29: 133-144.

Hau, M., R.E. Ricklefs, M. Wikelski, K.A. Lee and J.D. Brawn. 2010. Corticosterone, testosterone and life-history strategies of birds. Proc Biol Sci 277: 3203-3212.

Hayward, L.S. and J.C. Wingfield. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. Gen Comp Endocrinol 135: 365-371.

Hillgarth, N., M. Ramenofsky and J. Wingfield. 1997. Testosterone and sexual selection. Behav Ecol 8: 108-109.

Höhn, E.O. 1970. Gonadal hormone concentrations in northern phalaropes in relation to nuptial plumage. Can J Zool 48: 400-401.

Höhn, E.O. and S.C. Cheng. 1967. Gonadal hormones in Wilson`s phalaropes (*Steganopus tricolor*) and other birds in relation to plumage and sex behavior. Gen Comp Endocrinol 8: 1-11.

Holberton, R.L. and J.C. Wingfield. 2003. Modulating the corticosterone stress response: A mechanism for balancing individual risk and reproductive success in arctic-breeding sparrows? Auk 120: 1140-1150.

Huntoon, K.M., Y. Wang, C.A. Eppolito, K.W. Barbour, F.G. Berger, P.A. Shrikant and H. Baumann. 2008. The acute phase protein haptoglobin regulates host immunity. J Leukoc Biol 84: 170-181.

Jones, A.G. 2009. On the opportunity for sexual selection, the Bateman gradient and the maximum intensity of sexual selection. Evolution 63: 1673-1684

Kanda, N., T. Tsuchida and K. Tamaki. 1996. Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells. Clin Exp Immunol 106: 410-415.

Ketterson, E.D., V. Nolan Jr, L. Wolf, C. Ziegenfus, A.M. Dufty Jr, G.F. Ball and T.S. Johnsen. 1991. Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos. Horm Behav 25: 489-503.

Klein, S.L. 2000. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci Biobehav Rev 24: 627-638.

Klukowski, L.A., J.M. Cawthorn, E.D. Ketterson and V. Nolan Jr. 1997. Effects of experimentally elevated testosterone on plasma corticosterone and corticosterone-binding globulin in Dark-eyed Juncos (*Junco hyemalis*). Gen Comp Endocrinol 108: 141-151.

Liva, S.M. and R.R. Voskuhl. 2001. Testosterone acts directly on $CD4^+$ T lymphocytes to increase IL-10 production. J Immunol 167: 2060-2067.

Love, O.P., E.H. Chin, K.E. Wynne-Edwards and T.D. Williams. 2005. Stress hormones: A link between maternal condition and sex-biased reproductive investment. Am Nat 166: 751-766.

Lundqvist, M.L., D.L. Middleton, C. Radford, G.W. Warr and K.E. Magor. 2006. Immunoglobulins of the non-galliform birds: antibody expression and repertoire in the duck. Dev Comp Immunol 30: 93-100.

Maechler, M. 2012. diptest: Hartigan's dip test statistic for unimodality - corrected code. R package version 0.75-4. http://CRAN.R-project.org/package=diptest

Martin, A., E.A. Dunnington, W.B. Gross, W.E. Briles, R.W. Briles and P.B. Siegel. 1990. Production traits and alloantigen systems in lines of chickens selected for high or low antibody-responses to sheep erythrocytes. Poult Sci 69: 871–878.

Martínez, J., G. Tomás, S. Merino, E. Arriero and J. Moreno. 2003. Detection of serum immunoglobulins in wild birds by direct ELISA: A methodological study to validate the technique in different species using antichicken antibodies. Funct Ecol 17: 700-706.

Mateos, C. 2005. The subordination stress paradigm and the relation between testosterone and corticosterone in male ring-necked pheasants. Anim Behav 69: 249-255.

Meddle, S.L., N.T. Owen-Ashley, M.I. Richardson and J.C. Wingfield. 2003. Modulation of the hypothalamic-pituitary-adrenal axis of an Arctic-breeding polygynandrous songbird, the Smith's longspur, *Calcarius pictus*. Proc Biol Sci 270: 1849-1856.

Møller, A.P. 1995. Hormones, handicaps and bright birds. Trends Ecol Evol 10: 121.

Mousseau, T.A., T. Uller, E. Wapstra and A.V. Badyaev. 2009. Evolution of maternal effects: past and present. Philos Trans R Soc Lond Biol Sci 364: 1035-1038.

Muck, C. and W. Goymann. 2010. Throat patch size and darkness covaries with testosterone in females of a sex-role reversed species. Behav Ecol 22: 1312-1319.

Nunn, C.L., P. Lindenfors, E.R. Pursall and J. Rolff. 2009. On sexual dimorphism in immune function. Philos Trans R Soc Lond Biol Sci 364: 61-69.

O'Reilly, K.M. and J.C. Wingfield. 2001. Ecological factors underlying the adrenocortical response to capture stress in arctic-breeding shorebirds. Gen Comp Endocrinol 124: 1-11.

Owen-Ashley, N.T. and J.C. Wingfield. 2007. Acute phase responses of passerine birds: characterization and seasonal variation. J Ornithol 148: S583-S591.

Owen-Ashley, N.T., D. Hasselquist and J.C. Wingfield. 2004. Androgens and the immunocompetence handicap hypothesis: Unraveling direct and indirect pathways of immunosuppression in Song Sparrows. Am Nat 164: 490-505.

Poiani, A., A.R. Goldsmith and M.R. Evans. 2000. Ectoparasites of house sparrows (*Passer domesticus*): an experimental test of the immunocompetence handicap hypothesis and a new model. Behav Ecol Sociobiol 47: 230-242.

Pryke, S.R., L.A. Rollins, S.C. Griffiths and W.A. Buttemer. 2014. Experimental evidence that maternal corticosterone controls adaptive offspring sex ratio. Funct Ecol *In Press*

Quaye, I.K. 2008. Haptoglobin, inflammation and disease. Trans R Soc Trop Med Hyg 102: 735-742.

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Råberg, L., M. Grahn, D. Hasselquist and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. Proc Biol Sci 265: 1637-1641.

Rauw, W.M. 2012. Immune response from a resource allocation perspective. Front Genet 3: 267.

Roberts, M.L., K.L. Buchanan and M.R. Evans. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim Behav 68: 227-239.

Roberts, M.L., K.L. Buchanan, D. Hasselquist and M.R. Evans. 2007. Effects of testosterone and corticosterone on immunocompetence in the zebra finch. Horm Behav 51: 126-134.

Roberts, M.L., K.L. Buchanan, M.R. Evans, R.H. Marin and D.G. Satterlee. 2009. The effects of testosterone on immune function in quail selected for divergent plasma corticosterone response. J Exp Biol 212: 3125-3131.

Roitt, I., J. Brostoff and D. Male. 1993. Immunology, 3rd ed. Mosby, London.

Rolff, J. 2002. Bateman's principle and immunity. Proc Biol Sci 269: 867-872.

Romero, L.M. and J.C. Wingfield. 1998. Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's White-crowned sparrows (*Zonotrichia leucophrys gambelii*). Comp Biochem Physiol 119C: 31-36.

Roth, O., J.P. Scharsack, I. Keller and T.B.H. Reusch. 2011. Bateman's principle and immunity in a sex-role reversed pipefish. J Evol Biol 24: 1410-1420.

Sapolsky, R.M., M. Romero and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparatory actions. Endocr Rev 21: 55-89.

Schlinger, B.A., A.J. Fivizzani and G.V. Callard. 1989. Aromatase, 5α- and 5β-reductase in brain, pituitary and skin of the sex-role reversed Wilson's phalarope. J Endocrinol 122: 573-581.

Schoech, S.J., E.D. Ketterson and V. Nolan, Jr. 1999. Exogenous testosterone and the adrenocortical response in dark-eyed juncos. Auk 116: 64–72.

Sheldon, B.C. and S. Verhulst. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 11: 317–321.

Shen, H., Y. Song, C.M. Colangelo, T. Wu, C. Bruce, G. Scabia, A. Galan, M. Maffei and D.R. Goldstein. 2012. Haptoglobin activates innate immunity to enhance acute transplant reject mice. J Clin Invest 122: 383-387.

Siegel, P.B., W.B. Gross and J.A. Cherry. 1982. Correlated responses of chickens to selection for production of antibodies to sheep erythrocytes. Anim Blood Groups Biochem Genet 13: 291–297.

Silverin, B. and J.C. Wingfield. 1982. Patterns of breeding behavior and plasma levels of hormones in a free-living population of pied flycatchers, *Ficedula hypoleuca*. J Zool (1987) 198: 117-129.

Slater, C.H., M.S. Fitzpatrick and C.B. Schreck. 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen Comp Endocrinol 100: 218-225.

Swett, M.B. and C.W. Breuner. 2008. Interaction of testosterone, corticosterone and corticosterone binding globulin in the white-throated sparrow (*Zonotrichia albicollis*). Comp Biochem Physiol A Physiol 151: 226-231.

Tracy, D.M., D. Schamel and J. Dale. 2002. Red Phalarope (*Phalaropus fulicarius*). In: Poole, A. (Ed.), The Birds of North America Online. Cornell Lab of Ornithology, Ithaca (NY).

Trivers, R.L. 1972. Parental Investment and Sexual Selection. Aldine Press, Chicago.

Voigt, C. and W. Goymann. 2007. Sex-role reversal is reflected in the brain of African Black Coucals (*Centropus grillii*). Dev Neurobiol 67: 1560-1573.

Washburn, B.E., D.L. Morris, J.J. Millspaugh, J. Faaborg and J.H. Schulz. 2002. Using a commercially available radioimmunoassay to quantify corticosterone in avian plasma. Condor 104: 558–563.

Washburn, B.E., J.J. Millspaugh, D.L. Morris, J.H. Schulz and J. Faaborg. 2002. Using a commercially available enzyme immunoassay to quanitify testosterone in avian plasma. Condor 109: 181-186.

Williams, G.C. 1975. Sex and Evolution. Princeton University Press, Princeton.

Zuk, M. and K.A. McKean. 1996. Sex difference in parasite infections: patterns and processes. Int J Parasitol 26: 1009-1024.

CHAPTER 5: Condition and testosterone dependent plumage colouration and sexual dichromatism in a sex-role reversed bird, the Red phalarope.

Abstract

Elaborate ornaments are often used in mate assessment because they confer information about the phenotypic quality of the individual possessing the trait. The honest expression of condition-dependent plumage traits in birds is usually positively linked to testosterone levels in males. I assessed condition dependence of plumage colouration in Red phalaropes, a sex-role reversed shorebird where females compete for access to male mates, are more colourful than males and have lower levels of testosterone. I expected females in better body condition, and with superior indices of immune function, to have higher levels of testosterone and more impressive plumage. I found evidence for condition dependence in both female and male plumage traits, predominantly in terms of feather hue. Although there were sex specific relationships among condition indices and plumage traits, immune measures were consistently negatively related to plumage colour. Testosterone levels were only related to plumage colour of the primary coverts in females. I found the strongest condition dependence in breast plumage colour, and this plumage trait was also the most sexually dimorphic of the traits assessed in this study. These results may reflect a cost to producing colourful plumage, although the nature of these costs appears to be sex specific.

Introduction

Sexual selection leads to the evolution of secondary sexual characters because of the mating advantages afforded to those that possess these traits. In birds, males with brighter or more elaborate plumage are often of higher phenotypic or genetic quality. For example, more colourful birds are often in better condition (Keyser and Hill 1999, Estep *et al*. 2006), are more immunocompetent (e.g., Simons *et al*. 2012) and have fewer infections (Hill and Farmer 2004, del Cerro *et al*. 2010). Furthermore, females mated to males with more elaborate plumage can produce larger clutches or invest more in provisioning offspring (Sheldon 2000, Ratikainen and Kokko 2009). As a consequence, males accrue a fitness benefit via direct mate choice (e.g., Hill 1999) or higher rates of extra pair copulations (e.g., Yezerinac and Weatherhead 1997) because females prefer males with brighter and/or more elaborate plumage (Hill 2006, Kigma *et al*. 2008).

Sexual signals in the form of plumage traits should supply reliable information about the individual possessing the trait (Kodric-Brown and Brown 1984). That is to say, females assess male quality based on signal expression so that the signal should reliably predict individual quality. In order to achieve reliability, the honest expression of plumage traits has been conceptually linked to testosterone via the Immunocompetence Handicap Hypothesis (ICHH; Folstad and Karter 1992). The ICHH states that the immunosuppressive role of testosterone maintains signal honesty because high levels of testosterone produce a more elaborate trait and only individuals of high quality can afford the resulting high levels of immunosuppression. Thus, good quality individuals produce superior plumage traits that are used as viability indicators in mate choice.

Studies of sexual selection are typically focussed on systems where maleness, testosterone, and showy plumage are inextricably linked. Within those studies, the focus is

typically on male traits. One of the fundamental observations leading to the ICHH is that males tend to have high levels of testosterone and poorer immune function relative to females (Folstad and Karter 1992). However, female plumage can also be a reliable indicator of quality, even in species where males are brighter or similarly coloured (Piersma *et al*. 2001, Roulin *et al*. 2001, Siefferman and Hill 2005, Hanssen *et al*. 2006, Doutrelant *et al*. 2012). This suggests that high levels of testosterone alone are not a necessary condition for the reliable expression of condition-dependent plumage.

I investigated the relationships among the expression of plumage colouration, individual condition and testosterone in a sex-role reversed shorebird species, the Red phalarope (*Phalaropus fulicarius*). Phalaropes are sexually dichromatic with females possessing more colourful plumage than males. In particular, the reddish breast colour is more colourful in females, and females have a black cap that is not present in males, and which provides a notable contrast with the white facial feathers (Tracy *et al*. 2002). The back and wing colouration also is more saturated and has higher contrast in females.

Plumage colouration generally appears to be testosterone dependent in the Charadriiformes (Kimball and Ligon 1999, Muck and Goymann 2011) suggesting that a link between individual condition and plumage could be mediated by testosterone. Among phalaropes in particular, administering testosterone induces bright nuptial plumage characteristic of females in both males and females (Johns 1964). Yet subsequent work suggests that male phalaropes have typically higher levels of testosterone than females during the breeding season (Fivizzani *et al*. 1986, Chapter 4). This suggests that maleness, particularly in terms of overtly high levels of testosterone, may not be required for the reliable expression of plumage traits.

The main question of this study hinges upon the notion that if plumage represents a condition-dependent trait subject to sexual selection, then showier female and male phalaropes should be in better condition, particularly as measured by aspects of the immune system. I make several predictions based on this argument: 1) I predicted that traits that show sexual dichromatism are more likely to be condition-dependent. Sexual dichromatism probably results from mate preference for a colourful trait by one sex, with the exaggerated expression of that trait ultimately resulting in the costly production of the trait (Arnqvist and Rowe 2005). As such, sexually dichromatic traits should be condition-dependent, at least in the sex expressing the exaggerated form. Moreover, how these traits are dichromatic, in terms of lightness, UV reflectance, saturation and hue, will inform about how these traits relate to aspects of condition. 2) Indices of condition are positively related to plumage colour, specifically in light of prediction 1. 3) I also predict that plumage expression should be related to testosterone levels, so that individuals with higher levels of testosterone should exhibit exaggerated plumage colouration.

Methods

Field Data Collection

Red phalaropes (*Phalaropus fulicarius*) were trapped on the breeding grounds at East Bay Migratory Bird Sanctuary, Nunavut, Canada (N63°59'13.5" W81°41'48.3"). Fieldwork was carried out in 2009-2010, when I trapped males and females during the courtship period (prior to incubation) using a CO_2 powered net gun (Fly Dragon Technology Co., Ltd) as reported in Edwards and Gilchrist (2011) while they were aggregated in small flocks on early melt ponds.

Birds were weighed using a 100g Pesola scale. Tarsus length was measured using digital calipers (±0.01mm). I also collected a small blood sample via puncture of the ulnar vein. At the time of collection, a blood smear was taken and the remaining blood sample kept on ice (<6hrs)

until samples were centrifuged. Plasma samples were then frozen at -20°C until assays were done in the lab.

Laboratory Assays

I measured the amount of circulating immunoglobulins and leukocytes as indices of the immune system. Immunoglobulin Y (IgY) is an important aspect of humoral immunity that is also involved in the acquired immune response. IgY recognizes and binds to specific antigens to facilitate opsonisation and presentation of antigens that are subsequently destroyed either by phagocytic cells or via the complement cascade (Roitt *et al*. 1993; Lundqvist *et al*. 2006). I take high levels to indicate a more robust immune system. The IgY assay is described in more detail in Chapter 4. I assessed circulating immunoglobulin levels using an enzyme-linked immunosorbant assay (ELISA) based on Martínez *et al*. (2003).

I determined the relative density of circulating leukocytes from blood smears by counting the number of lymphocytes and heterophils per 10,000 erythrocytes. Lymphocytes are responsible for the production of antibodies and also facilitate cell-mediated immunity (Roitt *et al*. 1993). Heterophils are a phagocytic cell type that can function in a purely innate capacity or as an important component of cell-mediated and humoral immune responses (Roitt *et al*. 1993).

I assessed testosterone levels using a commercially available ELISA kit (Salimetrics, State College, Pennsylvania PA, USA) following the manufacturer's recommendations as reported in Chapter 4.

Plumage Colour Measurements

At the time of capture, I collected a small sample of feathers from locations that are likely avenues for sexual selection. I collected feathers (approximately 5) from the red breast plumage of both males and females and 5-10 feathers from the black crown of females (males lack the

black crown). I also took a single primary covert (3rd) to assess the white colouration of the wing. The breast and crown plumage colour of phalaropes is typical of that produced by the pigment melanin. More specifically, the reddish breast and black crown colouration are consistent with colours that result from high levels of phaeomelanin and eumelanin, respectively (McGraw 2006). White colouration, such as in the primary coverts, is due to light reflecting off the microstructure of the feather, rather than pigment-based colouration (Prum 2006). Phalaropes undergo a basic moult after departing the breeding grounds but prior to fall migration, at which time the primary coverts are moulted in (Tracy *et al*. 2002). Phalaropes obtain their alternate colouration of the breast and crown plumage in the spring prior to returning to the breeding grounds (Tracy *et al*. 2002). Therefore, depending on the plumage trait, I am assessing relationships between condition and plumage colour based on plumage that was moulted as much as 10 month prior to the study. Samples were stored in envelopes in a dry location for about 6 months until analyses were done in the lab. Feathers were mounted in a natural orientation on matte black paper and reflectance was measured using an Ocean Optics USB4000 (Dunedin FL, USA) spectrometer. I took 5 readings for each feather trait, and the average of these was used to represent the degree of reflectance relative to a white standard. I restricted the analysis to wavelengths of 300-700nm to match the part of the spectrum visible to birds (Pryke 2007).

Data Analysis

My approach was to determine whether testosterone was related plumage expression and indices of condition, taken as the immunological measures as well as 'body condition'. Body condition was taken to be the residuals of a regression of mass against body size for all individuals (hereafter called 'residual mass'), where body size was taken as the first axis from a principal

component analysis of wing, tarsus and culmen length. This axis explained 55% of the variation in size variables and all three variables contributed positively (eigenvectors >0.48 for all). I trapped 39 females and 40 males. All statistical analyses were performed in the R environment (R Core Team 2012) with support from the vegan package (Oksanen *et al*. 2013).

Nine spectral variables were returned by the software (OOIBase 32, Ocean Optics) representing variations of lightness, UV chroma, saturation and hue of feathers. However, given the qualities of the feather traits used in my study, these nine variables were reduced to the single most appropriate variable for each of lightness, UV chroma, saturation and hue (Personal Communication, Stephanie Doucet). Phalaropes are clearly sexually dichromatic. However, I was particularly interested in how sex differences in trait measures (i.e. lightness, UV, saturation, hue) might be used to make predictions about expectations of condition dependence determined from subsequent analyses. I used PCA scores of feather traits to determine sex differences in plumage colour using linear models and included date and year as factors. I did PCAs with males and females combined for breast and primary covert plumage and retained the first two axes because these included strong loadings of all the original variables.

To determine whether plumage traits were related to indices of condition and testosterone, I used Redundancy Analysis (RDA). RDA is a constrained ordination technique that describes multiple dependent variables (trait measures: lightness, UV chroma, saturation and hue) as a linear combination that best explains the independent variables (condition measures). The linear combination of dependent variables is expressed in terms of eigenvectors similar to traditional Principal Components Analysis (PCA). Sample sizes differed between some variables so I ran separate RDAs for IgY and residual mass, and for lymphocytes and heterophils and these are described as separate models for each plumage trait. Year and date were incorporated into

models as conditioning factors (cf. covariates). I have previously reported that testosterone titres are bimodally distributed in this sample of male phalaropes (Chapter 4) possibly because males with low testosterone were not yet in fully reproductive state. Consequently, I rank transformed testosterone values in males. Additionally, I standardized male values within the high and low testosterone groups and then re-ran analyses with all males together, however the interpretation of the results did not differ and so I report results from rank transformed data. Model terms were tested for significance using a permutation technique that returns F coefficients similar to ANOVA.

Results

PCA of Plumage Traits and Sexual Dichromatism

Based on principal components, I found notable differences in the sexes in only the second principal component for breast scores (PC2 breast: $t_{2,75}$=8.79, P<0.001, Year: $t_{2,75}$=-2.58, P=0.01; Table 9), whereby females had higher UV reflectance and hue (i.e., were redder) than males. There were non-significant tendencies for females to also have darker and more saturated breasts (PC1 breast: $t_{2,75}$=-1.67, P=0.10, Year: $t_{2,75}$=-4.61, P<0.001), and to have less saturated and greater UV reflectance in the primary coverts (PC1 primary covert: $t_{2,64}$=1.67, P=0.10, Year: $t_{2,64}$=-1.57, P=0.12). There was no difference between males and females in the second PC of primary covert plumage ($t_{2,64}$=-0.43, P=0.67, Year: $t_{2,64}$=2.02, P=0.05).

Condition Dependence of Plumage

The expression of breast and crown plumage in females was condition-dependent albeit in the opposite direction than predicted (results for Models 1 & 4 in Table 10). Females with less plasma IgY (i.e., lower immunity; Model 1) had breast plumage that was more saturated and redder (higher values for hue), and to a lesser extent was darker and had lower UV reflectance

(Table 11). Similarly, females with higher values for hue in their crown plumage had fewer heterophils (Table 11). Additionally, lymphocytes were also negatively related to breast plumage

Table 9. Loadings for the first two axes of Principal Component Analyses for each feather trait. These axes were used to investigate spectral differences between males in females in breast and primary coverts. Crown colour was not quantified for males and so was not used in the analysis of sex differences but the results of the PCA are presented nonetheless for females. For all three measures, lightness, UV chroma and saturation loaded heavily on the first axis, whereas hue loaded predominantly on axis 2.

	Breast		Primary Covert		Crown	
	PC1	PC2	PC1	PC2	PC1	PC2
% Variance Explained	65.2	23.1	56.2	25.6	51.7	25.4
Lightness	-0.531	-0.205	-0.437	-0.418	0.575	-0.320
UV Chroma	-0.454	0.656	-0.622	0.035	-0.629	0.230
Saturation	0.590	-0.165	0.627	-0.013	0.476	0.293
Hue	0.405	0.708	-0.168	0.908	0.217	0.871

(Model 2) and negatively related to primary covert colouration (Model 6) in models that had moderate effects but that were non-significant (Table 10). Although not statistically significant, the results from lymphocytes reflect a common tendency for plumage expression, particularly hue and UV chroma (see Models 2 & 6 in Table 11), to be negatively related to immune measures.

Among males, plasma IgY concentration was negatively related to primary covert colouration (Model 9 in Table 10). Hue contributed largely to this relationship (Table 11) but UV chroma and lightness were also negatively related and saturation was positively related. Although not significant, males with more lymphocytes had breast plumage that was less red, less saturated and with higher UV chroma and lightness (Table 11).

Table 10. Results of Redundancy Analyses for female (above) and male (below) plumage traits against condition indices. Separate models were run based on sample size among measures. Coefficients for plumage axes represent the ability of the condition measures to explain variation in lightness, UV chroma, saturation and hue of the plumage trait. The contribution of lightness, UV, saturation and hue to the axes are presented as eigenvectors in Table 11. P values were determined from randomized F values.

	Plumage Trait	Model	Term	DF	F	P
Females	Breast	Model 1	Plumage Axis	2,35	7.61	**0.005**
			IgY		6.57	**0.01**
			Residual Mass		1.04	0.31
		Model 2	Plumage Axis	2,25	2.02	0.15
			Lymphocytes		1.93	0.16
			Heterophils		0.09	0.84
	Crown	Model 3	Plumage Axis	2,35	2.48	0.11
			IgY		0.01	0.98
			Residual Mass		2.48	0.09
		Model 4	Plumage Axis	2,25	5.41	**0.02**
			Lymphocytes		0.59	0.58
			Heterophils		4.81	**0.03**
	Primary Covert	Model 5	Plumage Axis	2,31	0.71	0.40
			IgY		0.68	0.37
			Residual Mass		0.04	0.90
		Model 6	Plumage Axis	2,21	1.79	0.17
			Lymphocytes		1.77	0.14
			Heterophils		0.02	0.86
Males	Breast	Model 7	Plumage Axis	2,36	0.65	0.46
			IgY		0.55	0.45
			Residual Mass		0.10	0.83
		Model 8	Plumage Axis	2,24	3.00	0.09
			Lymphocytes		2.47	0.09
			Heterophils		0.52	0.47
	Primary Covert	Model 9	Plumage Axis	2,30	4.22	**0.05**
			IgY		3.86	**0.04**
			Residual Mass		0.36	0.59
		Model 10	Plumage Axis	2,19	0.09	0.78
			Lymphocytes		0.09	0.68
			Heterophils		0.01	0.97

Table 11. Eigenvectors representing the contribution of plumage traits to Plumage Axes from the Redundancy Analyses (RDA, Tables 1 & 2). I have included only those models with significant (bold) or marginal effects. The direction of the relationship between condition indices and the original plumage traits can be interpreted by the sign of the eigenvector.

Sex	**Female**	Female	Female	**Female**	Female	Male	**Male**	**Female**
Plumage Trait	**Breast**	Breast	Crown	**Crown**	Primary Covert	Breast	**Primary Covert**	**Primary Covert**
RDA Model	**Model 1**	Model 2	Model 3	**Model 4**	Model 6	Model 8	**Model 9**	**Testosterone**
Lightness	0.33	-0.24	-0.47	0.16	-0.08	0.31	-0.29	-0.14
UV Chroma	0.31	-0.43	-0.06	-0.04	-0.41	0.43	-0.38	-0.65
Saturation	-0.51	0.27	0.58	-0.11	0.34	-0.45	0.35	0.51
Hue	-0.56	-0.69	0.18	-0.65	-0.87	-0.52	-0.58	-0.82

Table 12. Results of Redundancy Analyses for female (above) and male (below) plumage traits against testosterone. The contribution of individual plumage measures to the plumage trait axes can be found in Table 11.

	Plumage Trait	DF	F	P
Females	Breast	10	0.35	0.65
	Crown	10	0.02	0.89
	Primary Covert	9	10.42	**0.01**
Males	Breast	12	0.42	0.55
	Primary Covert	9	0.82	0.36

Testosterone and Plumage Expression

The only support for a relationship between testosterone levels and plumage expression in either sex (Table 12) was in primary covert colouration in females. Female primary covert colouration was negatively related to testosterone levels, whereby females with more testosterone had plumage that had lower values for UV chroma and hue, and greater saturation (Table 11). Breast and crown colouration were not related to testosterone levels in females, nor were breast and primary covert colouration in males (Table 12).

Discussion

Sexual Dichromatism

I found statistically significant differences only in the hue and UV aspects of breast plumage, with non-significant tendencies for saturation and lightness. Consistent with my expectations, the hue of the breast plumage in females showed the strongest relationship to condition indices (i.e., IgY concentration), with a weak tendency observed in males. This pattern is consistent with the effect being muted in the sex expressing the less exaggerated plumage as well as the notion that dichromatic traits should be condition-dependent. However, the picture was not as clear with primary covert colouration. There was a non-significant tendency towards sexual dichromatism in primary covert colouration but the mild differences were most notable for saturation and UV

chroma, yet hue had the strongest relationship to condition indices in both sexes. Moreover, there was stronger support for condition dependence in males, rather than females, for this trait. Together, these results do not demonstrate a high degree of concordance between intra-sexual condition dependence and inter-sexual dichromatism.

Condition Dependence of Plumage

Several plumage traits of female and male phalaropes were related to measures of immunological condition, but in the opposite direction than predicted. From previous work in this field, more colourful (i.e., redder) plumage is typically more attractive to potential mates and is expected to indicate a superior mate (e.g., Estep *et al*. 2006, Kingma *et al*. 2008, del Cerro *et al*. 2010, Simons *et al*. 2012). However, I found that immunocompetence was negatively related to plumage traits in all relationships that were either significant or had non-significant trends (Table 11). Moreover, in all of these instances, hue was the predominant plumage measure involved in negative relationships I detected between plumage traits and immunocompetence. Since greater values of hue are achieved through the increased deposition of melanin pigmentation in plumage (McGraw *et al*. 2005), my findings suggest that a potential trade-off exists between the amount of pigment deposited in the plumage and immunocompetence in phalaropes. I speak to this in greater detail below.

Instances of negative condition dependence are uncommon but have been reported for various types of signalling traits (bill colour: Faivre *et al*. 2003; carotenoid-based plumage colour: Aguilera and Amat 2007, Vinkler *et al*. 2011). In some cases, negative relationships seem to result from trade-offs among immune components because a single plumage trait can be positively related to one immune measure, but negatively related to another (Møller and Petrie 2002, Garvin *et al*. 2008). However, in the present study I found only negative relationships

involving immunoglobulins (humoral and acquired immunity) and leukocytes (cell-mediated and innate immunity) demonstrating consistency across different arms of the immune system. An alternative explanation could be that the relationship between plumage expression and immunity changes from negative during the breeding season to positive in the non-breeding season (Gonzalez *et al*. 1999), particularly when the plumage is moulted in.

Although more colourful phalaropes may be inferior mates from the point of view of immunocompetence, redder females may be trading immune function against aspects of reproduction, such as egg production. Indeed, female plumage colouration can reflect egg and clutch traits in other bird species (Hanssen *et al*. 2009, Grindstaff *et al*. 2012). In a polyandrous species such as phalaropes, where the indirect benefits of mate choice are limited for males, female plumage expression may be strongly linked to aspects of egg production. A suitable mate for a male may then be a female who invests into eggs, i.e., produces large, well-provisioned eggs, at the expense of immunity. Indeed, there is a trade-off between antibody production and egg production in chickens (Siegel *et al*. 1982, Martin *et al*. 1990) suggesting that it may be prudent for polyandrous females to down-regulate immune function during egg production.

On that note, melanin-based female plumage traits could signal attributes of egg production to potential mates. The deposition of melanin in plumage can be facilitated by calcium via a number of physiological pathways (McGraw 2008). In Zebra finches (*Taeniopygia guttata*), supplementing the diet with calcium increases the expression of melanic plumage (McGraw 2007), and there is a relationship between the melanin content in feathers and the amount of calcium present in feathers and bones (Niecke *et al*. 2003, Roulin *et al*. 2006, but see Stewart and Westneat 2010). To my knowledge, there are no studies linking melanin-based plumage expression to the calcium requirements for other physiological processes, such as egg

formation, but the link seems apt given the connection between bone calcium stores and egg production in birds (Whitehead 2004). Likewise, tyrosine is an essential amino acid that is both limiting for the formation of egg proteins (Murphy 1994) and is necessary for the synthesis of melanin pigments (Simon *et al*. 2009). Females may then signal fecundity via melanin-based plumage traits (Vergara *et al*. 2009). Thus, calcium stores or essential amino acids could underlie melanin-based plumage expression, which may ultimately convey information about egg quality and production to prospective mates.

Testosterone and Plumage Expression

I did not find much evidence to suggest that plumage expression is related to testosterone levels in either sex. However, I did find that the colouration of the primary coverts in females was related to testosterone; females with lower testosterone had plumage with higher UV reflectance and hue. This finding is also contrary to my predictions because birds with higher testosterone levels often have more elaborate, not less elaborate, plumage (e.g., Gonzalez *et al*. 2001, Roberts *et al*. 2009, Lindsay *et al*. 2011), and this is true for females of another sex-role reversed species (Muck and Goymann 2011). Despite a lack of direct evidence of a relationship between plumage colouration and testosterone, I did find that females with more brightly coloured breasts had lower IgY levels, and, in Chapter 4, that females with higher IgY had lower levels of testosterone, possibly suggesting an indirect link between these traits.

In tropical buttonquail (*Turnix suscitator*), there was considerable inter-individual variation in testosterone from month to month (Muck and Goymann 2011). The authors reported a correlation between melanin-based plumage and testosterone using average annual testosterone levels calculated for each individual, but it is plausible that using a single time point may not have yielded a significant relationship given the degree of variation. In the present study, I

assume that testosterone at the time of moult - approximately 3 months prior to when the samples were collected for crown and breast plumage, and 8 months prior for primary coverts - is related to testosterone measured during the breeding season, when in fact enough variation between the two times could obscure a meaningful relationship, particularly at relatively low sample size.

Conclusion

This study is one of the first to test for relationships between plumage expression and immune function and testosterone levels in a sex-role reversed species. Despite demonstrating that signals reliably encoded information about the signaller, contrary to my prediction, there was a negative relationship between immune components and black crown plumage, red breast plumage and white plumage of the primary coverts. Although precisely which feather trait related to which immune measure differed between the sexes, feather hue was consistently the most important spectral measurement explaining immune function. Whereas this pattern is consistent with a cost of pigment deposition in feathers, and the notion of an immunocompetence handicap, negative relationships between plumage expression and indices of condition are uncommon. The signalling dynamics of sex-role reversed species are poorly studied; it is possible that the unique demands placed upon polyandrous females accounts for the negative relationship if unmeasured traits involved in mate choice, such as egg production, were involved with trade-offs with immune function during the breeding season. However, whereas these trade-offs could occur in females they do not explain negative relationships I also observed in males, and so serves to highlight that there are still considerable gains to be made in understanding the mechanisms behind reliable signalling, as well as the variation among species in how condition relates to plumage signals.

Literature Cited

Aguilera, E. and J.A. Amat. 2007. Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). Naturwissenschaften 94: 895-902.

Arnqvist G. and L. Rowe. 2005. Sexual Conflict. Princeton: Princeton University Press.

del Cerro, S., S. Merino, J. Martínez-de la Puente, E. Lobato, R. Ruiz-de-Castañeda, J. Rivero-de Aguilar, J. Martínez, J. Morales, G. Tomás and J. Moreno. 2010. Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*). Oecologia 162: 825-835.

Doutrelant, C., A. Grégoire, A. Midamegbe, M. Lambrechts and P. Perret. 2012. Female plumage coloration is sensitive to the cost of reproduction. An experiment in blue tits. J Anim Ecol 81: 87-96.

Edwards, D.B. and H.G. Gilchrist. 2011. A new means of catching shorebirds: the Super Talon net gun. Wader Study Group Bulletin 118: 134-6.

Estep, L.K., M.D. Shawkey and G.E. Hill. 2006. Carotenoid-based breast plumage colour, body condition and clutch size in red foodies (*Foudia madagascariensis*). Ostrich 77: 164-169.

Faivre, B., M. Préault, F. Salvadori, M. Théry, M. Gaillard and F. Cézilly. 2003. Bill colour and immunocompetence in the European blackbird. Animal Behaviour 65: 1125‑1131.

Fivizzani, A.J., M.A. Colwell and L.W. Oring. 1986. Plasma steroid hormone levels in free-living Wilson's Phalaropes, *Phalaropus tricolor*. Gen Comp Endocrinol. 62: 137-144.

Folstad, I. and A.J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603-622.

Garvin, J.C., P.O. Dunn, L.A. Whittingham, D.A. Steeber and D. Hasselquist. 2008. Do male ornaments signal immunity in the common yellowthroat? Behav Ecol 19: 54-60.

Gonzalez, G., G. Sorci and F. de Lope. 1999. Seasonal variation in the relationship between cellular immune response and badge size in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 46: 117‑122.

Gonzalez, G., G. Sorci, L.C. Smith and F. de Lope. 2001. Testosterone and sexual signalling in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 50: 557–562.

Grindstaff, J.L., M.B. Lovern, J.L. Burtka and A. Hallmark-Sharber. 2012. Structural coloration signals condition, parental investment, and circulating hormone levels in Eastern bluebirds (*Sialia sialis*). J Comp Physiol A 198: 625-637.

Hanssen, S.A., I. Folstad and K.E. Erikstad. 2006. White plumage reflects individual quality in female eiders. Anim Behav 71: 337-343.

Hanssen, S.A., J.O. Bustnes, T. Tveraa, D. Hasselquist, O. Varpe and J.A. Henden. 2009. Individual quality and reproductive effort mirrored in white wing plumage in both sexes of south polar skuas. Behav Ecol 20: 961-966.

Hill, G. E. 1999. Mate choice, male quality, and carotenoid-based plumage coloration. In: Adams, N. and R. Slowtow (Eds.), Proc 22 Int. Ornithol. Congr. University of Natal, Durban. pp. 1654-1668.

Hill, G. E. 2006. Female mate choice for ornamental plumage. In: Hill, G.E. and K.J. McGraw (Eds.), Bird Coloration, Vol II: Function and Evolution. Harvard University, Cambridge. pp. 137-200.

Hill, G.E. and K.L. Farmer. 2004. Carotenoid-based plumage coloration predicts resistence to a novel parasite in the house finch. Naturwissenschaften 92: 30-34.

Johns, J.E. 1964. Testosterone-induced nuptial feathers in phalaropes. Condor 66: 449-455.

Keyser, A.J. and G.E. Hill. 1999. Condition-dependent variation in the blue-ultraviolet coloration of a structurally based plumage ornament. Proc Biol Sci 266: 771-777.

Kingma, S.A., I. Szentirmai, T. Székely, V. Bókony, M. Bleeker, A. Liker and J. Komdeur. 2008. Sexual selection and the function of a melanin-based plumage ornament in polygamous penduline tits *Remiz pendulinus*. Behav Ecol Sociobiol 62: 1277-1288.

Kimball, R.T. and J.D. Ligon. 1999. Evolution of avian plumage dichromatism from a proximate perspective. Am Nat 154: 182-193.

Kodric-Brown, A. and J.H. Brown. 1984. Truth in advertising: the kinds of traits favored by sexual selection. Am Nat 124: 309-323.

Lindsay, W.R., M.S. Webster and H. Schwabl. 2011. Sexually selected male plumage color is testosterone dependent in a tropical passerine bird, the Red-backed Fairy-Wren (*Malurus melanocephalus*). PLoS ONE 6(10): e26067.

Lundqvist, M.L., D.L. Middleton, C. Radford, G.W. Warr and K.E. Magor. 2006. Immunoglobulins of the non-galliform birds: antibody expression and repertoire in the duck. Dev Comp Immunol 30: 93-100.

Martin, A., E.A. Dunnington, W.B. Gross, W.E. Briles, R.W. Briles and P.B. Siegel. 1990. Production traits and alloantigen systems in lines of chickens selected for high or low antibody-responses to sheep erythrocytes. Poult Sci 69: 871–878.

Martínez, J., G. Tomás, S. Merino, E. Arriero, and J. Moreno. 2003. Detection of serum immunoglobulins in wild birds by direct ELISA: A methodological study to validate the technique in different species using antichicken antibodies. Func Ecol 17: 700-706.

McGraw, K.J. 2006. The mechanics of melanin coloration in birds. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 243–294

McGraw, K.J. 2007. Dietary mineral content influences melanin-based ornamental coloration. Behav Ecol 18: 137-142.

McGraw, K.J. 2008. An update on the honesty of melanin-based color signals in birds. Pigment Cell Melanoma Res 21: 133-138.

McGraw, K.J., R.J. Safran and K. Wakamatsu. 2005. How feather colour reflects its melanin content. Func Ecol 19: 816-821.

Møller, A.P. and M. Petrie. 2002. Condition dependence, multiple sexual signals, and immunocompetence in peacocks. Behav Ecol 13: 248-253.

Morales, J., J. Moreno, S. Merino, G. Tomás, J. Martínez and Z. Garamszegi. 2004. Associations between immune parameters, parasitism, and stress in breeding pied flycatcher (*Ficedula hypoleuca*). Can J Zool 82: 1484-1492.

Muck, C. and W. Goymann. 2011. Throat patch size and darkness covaries with testosterone in females of a sex-role reversed species. Behav Ecol 22: 1312-1319.

Murphy, M.E. 1994. Amino acid composition of avian eggs and tissues: Nutritional implications. J Avian Biol 25: 27-38.

Niecke, M., S. Rothlaender and A. Roulin. 2003. Why do ornaments signal individual quality? Insights from metal element analysis of barn owl feathers. Oecologia 137: 153-158.

Oksanen, J., F.G. Blanchet, R. Kindt, P. Legendre, P.R. Minchin, R.B. O'Hara, G.L. Simpson, P. Solymos, M.H.H. Stevens and H. Wagner. 2013. vegan: Community Ecology Package. R package version 2.0-6. http://CRAN.R-project.org/package=vegan.

Piersma, T., L. Mendes, J. Hennekens, S. Ratiarison, S. Groenewold and J. Jukema. 2001. Breeding plumage honestly signals likelihood of tapeworm infestation in females of a long-distance migrating shorebird, the bar-tailed godwit. Zoology 104: 41-48.

Prum, K.J. 2006. Anatomy, physics, and evolution of structural colors. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 295–353.

Pryke S. R. 2007. Sexual selection of ultraviolet and structural color signals. In: Jamieson, B.M. (Ed), Reproductive Biology and Phylogeny of Aves. Science Publishers Inc., Enfield, USA. pp. 1-40.

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Ratikainen, I.I. and H. Kokko. 2010. Differential allocation and compensation: who deserves the silver spoon? Behav Ecol 21: 195-200.

Roberts, M.L., E. Ras and A. Peters. 2009. Testosterone increases UV reflectance of sexually selected crown plumage in male blue tits. Behav Ecol 20: 535–541.

Roitt, I., J. Brostoff and D. Male. 1993. Immunology, 3rd ed. London: Mosby.

Roulin, A., C. Riols, C. Dijkstra and A.-L. Ducrest. 2001. Female plumage spottiness signals parasite resistance in the barn owl (*Tyto alba*). Behav Ecol 12: 103-110.

Roulin, A., T. Dauwe, R. Blust, M. Eens and M. Beaud. 2006. A link between eumelanism and calcium physiology in the barn owl. Naturwissenschaften 93: 426-430.

Sheldon, B.C. 2000. Differential allocation: tests, mechanisms and implications. Trends Ecol Evol 15: 397-402.

Siefferman, L. and G.E. Hill. 2005. Evidence for sexual selection on structural plumage coloration in female Eastern bluebirds. Evolution 59: 1819-1828.

Siegel, P.B., W.B. Gross and J.A. Cherry. 1982. Correlated responses of chickens to selection for production of antibodies to sheep erythrocytes. Anim Blood Groups Biochem Genet. 13: 291–297.

Simon, J.D., D. Peles, K. Wakamatsu and S. Ito. 2009. Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res 22: 563-579.

Simons, M.J.P., A.A. Cohen and S. Verhulst. 2012. What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds – A meta-analysis. PLoS ONE 7: e43088.

Stewart, I.R.K., and D.F. Westneat. 2010. Dietary negatively affects the size of a status signal in juvenile male house sparrows (*Passer domesticus*). Auk 127: 549-557.

Tracy, D.M., D. Schamel and K. Dale. 2002. Red Phalarope (*Phalaropus fulicarius*). In: Poole, A. (Ed), The Birds of North America Online. Ithaca, Cornell Lab of Ornithology.

Vergara, P., J.A. Fargallo, J. Martínez-Padilla and J.A. Lemus. 2009. Inter-annual variation and information content of melanin-based coloration in female Eurasian kestrels. Biol J Linnean Soc 97: 781-790.

Vinkler, M., J. Schnitzer, P. Munclinger and T. Albrecht. 2011. Phytohaemaglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Anim Behav 83: 17-23.

Whitehead, C.C. 2004. Overview of bone biology in the egg-laying hen. Poult Sci 83: 193-199.

Yezerinac, S.M. and P.J. Weatherhead. 1997. Extra-pair mating, male plumage coloration and sexual selection in yellow warblers (*Dendroica petechia*). Proc Biol Sci 264: 527-532.

CHAPTER 6: On the fringe of mutual mate choice: positive plumage, and negative size, assortative mating in a polyandrous shorebird.

Abstract

In a sex-role reversed species, where females have higher reproductive potential than males and males provide all the parental care, the dominant paradigm portrays the choosy male exerting choice over potential female mates. On the other hand, females should not be choosy and should seek to mate with any and all males. However, in Red phalaropes (*Phalaropus fulicarius*), a polyandrous shorebird where females possess colourful plumage and seek multiple mates, several aspects of female behaviour suggest otherwise, including observations that pair formation can be initiated by either sex. I looked for patterns of assortative mating within pairs of the polyandrous Red phalarope and found strong evidence that mutual mate choice occurs in this system, where theory would not predict it. I found that males and females with redder breasts tended to mate together. I also found that generally larger females were mated to generally smaller males. Furthermore, in an independent sample of males, smaller males were found to be incubating larger eggs. My results are consistent with mutual mate choice rather than passive processes and I discuss how the traits measured in this study might be used in mutual mate choice. My results demonstrate that assortative mating occurs over a broader range of mating conditions than what might be otherwise expected and I discuss the implications for sexual selection and sexual conflict.

Introduction

The classical view of mate choice contrasts the 'choosy' female with males that mate indiscriminately. Indeed, this is the likely explanation for secondary sexual traits possessed by males: females prefer elaborate traits and use the expression of these traits to determine suitable males with which to mate (Andersson 1994). More recently, the expression of showy traits in both males and females has been suggested to reflect mutual sexual selection for these traits by both sexes (Amundsen 2000, Kraaijeveld *et al*. 2007), as opposed to their expression simply resulting from a genetic correlation among male and female traits.

Among birds, many studies of mutual mate choice have focussed on species that are largely monochromatic and/or monomorphic, as well as in those with biparental care (reviewed in Kraaijeveld *et al*. 2007; Bortolotti *et al*. 2008, Silva *et al* 2008, Griggio *et al*. 2009, Ledwoń 2011, Rowe and Weatherhead 2011). Mutual expression of traits is thought to be the result of mutual mate choice (Amundsen 2000, Kraaijeveld *et al*. 2007), and inequalities in parental care are thought to create situations in which mutual mate choice is unlikely (Johnstone *et al*. 1996, Kokko and Johnstone 2002). Thus, theory suggests that mutual mate choice and assortative mating should be more prevalent in monogamous species, and species that are largely sexually monomorphic. As a aconsequence of the theoretical framework, little empirical work has been done outside of monogamous species, but work outside of birds attests to novel conditions under which mutual mate choice can occur, including sexually dimorphic and polygamous species (Bonduriansky 2001, Berglund *et al*. 2005, Bel-Venner *et al*. 2008). Instances such as these, where empirical findings conflict with our theoretical predictions, highlight our lack of knowledge and stress the importance of understanding mutual processes in sexual selection (Jones and Ratterman 2009) to gain an appreciation of the interactions between natural selection, sexual selection and sexual conflict.

Here, I tested for assortative mating in a 'role-reversed' shorebird, the Red phalarope (*Phalaropus fulicarius*). Red phalaropes are regarded as serially polyandrous (Tracy *et al*. 2002); females lay eggs and males provide all subsequent parental care, while the female seeks another mate. Although realized rates of polyandry may be relatively low to moderate (Schamel and Tracy 1977, Whitfield 1995), the reproductive potential of females is clearly much higher than that of males in a given year. Female phalaropes possess the hallmarks of directional mate choice, most notably they have brighter and more contrasting plumage than males, and there is also a large degree of female-biased sexual size dimorphism (Tracy *et al*. 2002). Sexual dichromatism in species bearing testosterone-mediated traits, such as phalaropes (Johns 1964), is thought to occur as a result of selection for the exaggerated expression of the trait (Kimball and Ligon 1999). Therefore, the general expectation is that there is male choice for female mates, but not female choice for male mates.

Nonetheless, this study system has interesting characteristics that could allow for mutual mate choice. First, Red phalaropes are gregarious (Tracy *et al*. 2002). This reduces mate searching costs, increases encounter rates and ultimately permits the simultaneous assessment of multiple potential mates which can facilitate mutual mate choice (Kokko and Johnstone 2002, Barry and Kokko 2010). Additionally, neither sex is territorial (Tracy *et al*. 2002), which not only allows more time for mate assessment, because no time is devoted to defending territories, but this also enables females to travel to locate alternative mates. Pair formation is typically, but not necessarily, initiated by females; males can instigate pair formation (Tracy and Schamel 1988). Likewise, both males and females can initiate aerial chase flights undertaken during courtship (Schamel and Tracy 1977, Tracy and Schamel 1988), further suggesting that pairing can be bidirectional. Similarly, the pair bond is protected and reinforced via aggression directed

towards other phalaropes that approach too closely within the flock. Both males and females participate in the aggressive defence of the pair (Tracy *et al*. 2002; personal observation), reinforcing the idea that both sexes have interests in their mating partner.

Despite having clear inequalities in parental care and being sexually dimorphic, I hypothesize that that the unique behaviours observed in the polyandrous Red phalarope evidence mutual investment in mating decisions consistent with mutual mate choice. I predicted that mate choice in phalaropes may be mutual and based on both plumage colour and body size because phalaropes appear to have the opportunity for mate choice, and previous studies show that both sexes can initiate and protect pair bonds (Schamel and Tracy 1977, Tracy and Schamel 1988). I predicted the occurrence of mutual mate choice despite clear inequalities in reproductive potential and parental care, conditions thought to indicate a lack of opportunity for mutual mate choice to occur (Johnstone *et al*. 1996, Kokko and Johnstone 2002). I expected positive assortative mating in plumage colour traits because brighter, more colourful, plumage is often preferred in mate choice situations (Andersson 1994). I also expected larger individuals to mate amongst themselves, assuming that it is advantageous to be paired to a larger mate for both males and females. Larger females should lay larger, and therefore better, eggs (e.g., Grant 1991, Göth and Evans 2004), and so should be more desirable as mates to males. Likewise, larger males should be more effective incubators for larger eggs (Lislevand and Thomas 2006) and so should be desirable to females.

Methods

Study Population and Field Data Collection

I trapped Red phalaropes at East Bay Migratory Bird Sanctuary, Nunavut, Canada (N63° 59’ W81° 41’) in the summers of 2009 and 2010. Pre-incubation birds were caught using a CO_2

powered net gun (Fly Dragon Technology Co., Ltd) as described in Edwards and Gilchrist (2011) which allowed me to target and ultimately catch both members of the pair simultaneously. I trapped pairs during the courtship period while birds were aggregated in small flocks on early melt ponds. Within these flocks, paired males and females often show a clear association to each other, giving contact calls and becoming aggressive when other phalaropes approach too closely (Tracy *et al*. 2002). Birds were observed prior to being trapped to ensure they exhibited behaviours indicative of pairing. Birds were considered to be paired (n=20 pairs were trapped for this study) if they were mutually associating together within a flock (i.e. giving contact calls), and were aggressively chasing off other phalaropes that approached the pair too closely. 'Pairs' that did not at least exhibit pair defense behaviours were not trapped for this study. In 5 cases, I could confirm pairings by direct observations of copulation events. An independent sample of incubating males was trapped throughout the incubation period using bownets placed on the nest (Chapter 3; Edwards *et al*. 2013). Incubating males were trapped in order to relate the size of the incubating male to their eggs, as phalaropes may mate assortatively based on body size to facilitate incubation (see Lislevand and Thomas 2006).

My effort to capture paired birds was focussed early in the breeding season to reduce the probability of including previously mated birds in my sample because pairing dynamics can be influenced by prior reproductive attempts within a season in other species of phalaropes (Schamel *et al*. 2004). Paired birds were trapped predominantly in the last half of June (Julian Day 162-180) and incubating males were trapped while nests were active from late June to mid July (Julian Day 172-198). Courtship males and incubating males were different individuals. Through the course of this thesis, I trapped 40 paired or unpaired males during the courtship

period and in only one case did I find one of these males incubating a clutch within my 12km^2 study area (n=48 nests).

I measured mass of captured birds using a 100g Pesola scale. Tarsus and culmen lengths were measured using digital calipers (±0.01mm), and flattened wing chord with a 30cm wing rule. Feathers were collected as part of a larger study and analyzed after the field work was completed using a lab-based spectrophotometer (Ocean Optics USB4000; see Chapter 5 for methodology) to assess levels of hue, saturation, lightness and UV chroma. Feathers were collected from the crown, breast and primary coverts. Crown and breast colours are consistent with melanin-derived plumage (McGraw 2006) and are both moulted in spring prior to arriving on the breeding grounds (Tracy *et al*. 2002). The primary covert colour is structurally derived (Prum 2006) and these feathers are moulted in prior to the autumn migration the previous year (Tracy *et al*. 2002).

Nests were located by searching within a 3x4km study area. For nests found during the laying sequence, I returned on a daily basis to determine the date of clutch completion and the initiation of incubation. However, most nests were found well after laying was complete. For these nests, I used an egg flotation technique to estimate lay date (Liebezeit *et al*. 2007).

I measured length and width of eggs using digital calipers (±0.01mm) for all eggs of males caught while incubating. I was particularly interested in how metrics of general egg size could inform about the female that laid them, and so I averaged measures across all eggs in the nest. I calculated egg volume based upon length and width measures (following Narushin 2005). I used egg volume rather than egg mass because eggs lose mass as they develop and I could not sample at a consistent developmental time for all nests.

Data Analysis

My approach was to determine whether pairs mate assortatively based on plumage and morphometric traits. I also looked for evidence of size assortative mating in associations between incubating males and the size of the eggs they were incubating. All statistical analyses were performed in R (R Core Team 2012).

Because males and females may not mate assortatively on a trait by trait basis, I used a multivariate approach to identify the most important traits explaining mating patterns. I determined patterns of assortative mating using a constrained ordination technique, Canonical Correlation Analysis (CCA) using the R package CCA (González and Déjean 2009). CCA determines the linear combination of 'female' traits that best explains a linear combination of 'male' traits. I tested for significance (α=0.05) of the CCA axes using Roy's Largest Root method following a permutation procedure using the CCP package (Menzel 2011). However, because this technique returns atypical coefficients, I report only the p values for the significance tests, but I also include canonical roots (equivalent to an R-square value based on the canonical correlations) for the first canonical variates. CCA is quite conservative at small sample sizes (n<50; Hair *et al*. 2009), and so will only detect significance for extremely strong relationships at my sample size (n=20 pairs). Given the conservative nature of CCA, I used the technique as an exploratory procedure (Hair *et al*. 2009) to inform which variables to subsequently test using traditional correlation analyses. I limited my interpretation of important variables to those with the largest eigenvectors in the first canonical axes. All data used in CCA were scaled by year to remove year effects.

I previously identified condition dependence in phalarope plumage traits (Chapter 5). Specifically, hue was the spectral measurement that best reflected individual differences in condition in this population of Red phalaropes (see Tables 9 & 10, Chapter 5). Therefore to

reduce the number of plumage variables in the present study, I focussed the analysis to the PC axes where hue loads heavily for breast, crown and primary covert feathers (see Table 12, Chapter 5).

As a means of confirming CCA based on physical size measurements, I looked for evidence of assortative mating using male incubator morphometrics and average egg size. Specifically, I used male traits that correlated highly with female traits based on loadings from the size CCA in correlations with egg size, as well as the first principal component of all male size measures as a measure of overall body size. Here, I make the assumption that larger females lay larger eggs given that I could not get measurements from laying females. This assumption is reasonable considering the positive relationship that exists between female body and egg size previously reported in other shorebirds (e.g., Nol *et al*. 1984, Redmond 1986, Jönsson 1987, Blomqvist and Johansson 1995, Nol *et al*. 1997), as well as in other species of shorebirds at my site (unpublished data).

Relationships from traditional correlations are expressed as partial correlations including year as a term in the analyses. Partial correlation analyses were done using the ppcor package in R (Kim 2012). I expected the opportunity for mate choice to decline as the breeding season progressed due to a reduction in the availability of potential mates. As a consequence, I investigated the role that date played on mate choice mechanisms in two ways. First, I included date in the previous correlation models to determine to what degree date within the courtship period influenced pairings. I did this to differentiate between a direct mechanism of mate choice, where mate choice occurs through active mate selection, and a passive mechanism. As an example of a passive process, arrival time and plumage are frequently condition-dependent (e.g., Møller 1994) and as a result, higher quality males may already be paired and so be unavailable to

later arriving females. In the second approach, I took the residuals from the relationship between male and female traits as a measure of the variability in pairing dynamics. I did a similar analysis in incubating males and egg characteristics, but using lay date estimated from float regressions. If mate choice is relaxed later in the season, then the relationship between the residuals and trap/lay date should be heteroscedastic. I tested for heteroscedasticity in these relationships using the Breusch-Pagan test from the R package lmtest (Zeileis and Hothorn 2002).

Results

Assortative Mating based on Plumage Colour

The first canonical variates of plumage colour were explained mostly by PC2 of male breast and PC2 of female breast and PC2 of primary covert colour (Table 13) suggesting that these traits may be important for mate choice. The relationship between the first canonical variates was not significant using a multivariate approach (Roy's Largest Root method; P=0.34) although the correlation among the first canonical variates explained 48% of the variation retained by these axes suggesting breast plumage and female primary covert colour could play a role in explaining assortative mating. Using a traditional correlation approach of the main variables observed to load heavily on the first canonical variates of the CCA (Breast PC2 for both males and females; Table 13), the relationship between male and female breast colour approached significance ($r_{partial}$=0.40, P=0.08; year: $r_{partial}$=-0.02, P=0.98), however the relationship was constrained by the presence of an outlier (>2.5sd). This outlier was a pairing between a dull male and an exceptionally colourful female. With this single point removed, the relationship between male and female breast colouration was strong (n=19, $r_{partial}$=0.59, P=0.008; Fig. 6); more colourful males were paired with more colourful females based on breast plumage. The relationship between female primary covert colour (Pcov PC2) and male breast colour (Breast PC2) approached significance ($r_{partial}$=-0.42, P=0.055; year: $r_{partial}$=-0.02, P=0.95), demonstrating that

there was a tendency for males with more colourful breasts to be paired with females with less colourful and lighter (i.e., whiter) coverts.

Table 13. Loadings of standardized plumage colour traits on the first canonical variate for male and female phalaropes. Essentially, males with greater hue in their breast plumage were paired to females that also had greater breast hue, but less hue in their primary coverts. The loadings for the PC axes are presented in Table 12 of Chapter 5.

	Females	Males
Crown PC2	-0.049	-
Breast PC1	0.080	-0.353
Breast PC2	0.667	0.741
Pcov PC2	-0.952	0.166

Table 14. Loadings of standardized size measures on the first canonical variate for male and female phalaropes. Females with shorter wings and greater mass were paired with males with longer culmens.

	Females	Males
Wing	0.816	0.171
Tarsus	0.187	-0.038
Culmen	-0.370	-1.035
Mass	-0.511	-0.052

Assortative Mating based on Body Size

The first canonical variates of size were predominantly explained by male culmen and female wing, and to a lesser extent by female body mass (Table 14). The CCA model approached significance (P=0.10) and the correlation among the first canonical variates explained 65% of the variation retained in those axes. Again, I investigated the relationship among the main

components of the first canonical variates using standard correlation analysis of values adjusted for year differences. There was a strong negative correlation between female wing size and male culmen ($r_{partial}$=-0.64, P<0.001; year: $r_{partial}$=0.23, P=0.32; Fig. 7). Generally, larger females (i.e., females with larger wings) were paired to smaller males (i.e., smaller culmens). However, because there was an appreciable negative relationship between female mass and female wing size (Table 14), I repeated the analysis to include female mass. The inclusion resulted in a notable increase in the strength of the relationship (wing: $r_{partial}$=-0.74, P<0.001; mass: $r_{partial}$=0.51, P=0.02; year: $r_{partial}$=0.14, P=0.57). Thus, the revised analysis demonstrates that females with longer wings relative to their mass were mated to smaller males (i.e., smaller culmens). It should be pointed out that female wing size and body mass were poorly correlated using the original variables (r=0.09), and so the negative relationship between wing size and mass in paired females appears to be the product of mate selection rather than a natural relationship between the two traits.

If there is positive assortative mating based on breast plumage and negative assortative mating based on morphometrics, then it is conceivable that these traits might be correlated within a sex and that assortative mating proceeds directly through only plumage or morphometric traits. I ran a partial correlation including male culmen and breast colour, female wing and breast colour and year and found that pairing based on these traits does in fact occur independently. Both relationships remained significant with all terms in the model (male culmen vs female wing: $r_{partial}$=-0.56, P=0.01; male breast vs female breast: $r_{partial}$=0.63, P=0.003; with plumage outlier removed).

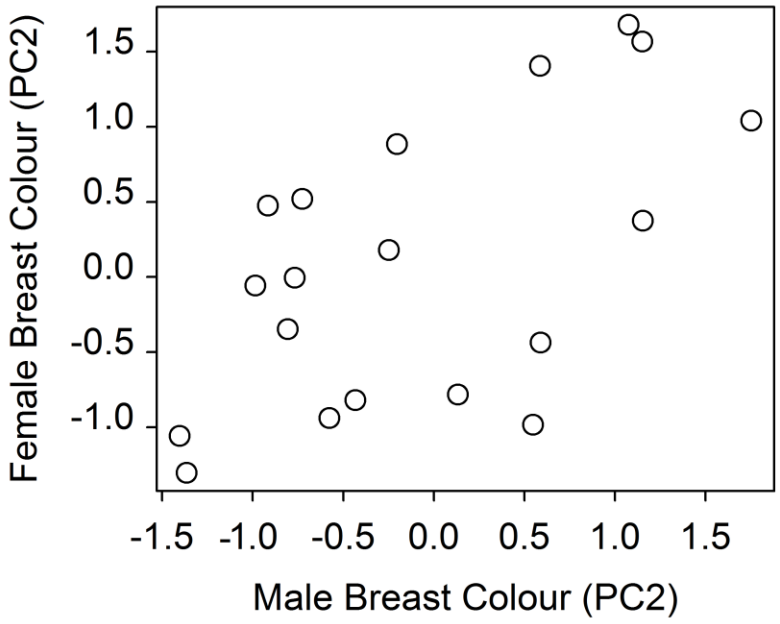


Fig. 6. Phalaropes displayed positive assortative pairing with respect to breast colouration. PC2 of breast colour was predominantly explained by feather hue (Chapter 5), such that redder coloured individuals were paired together. Based on the results of the CCA, hue of the white component of the primary coverts was also related to pairing but in that case, redder males were paired with females with lower values for hue (not shown).

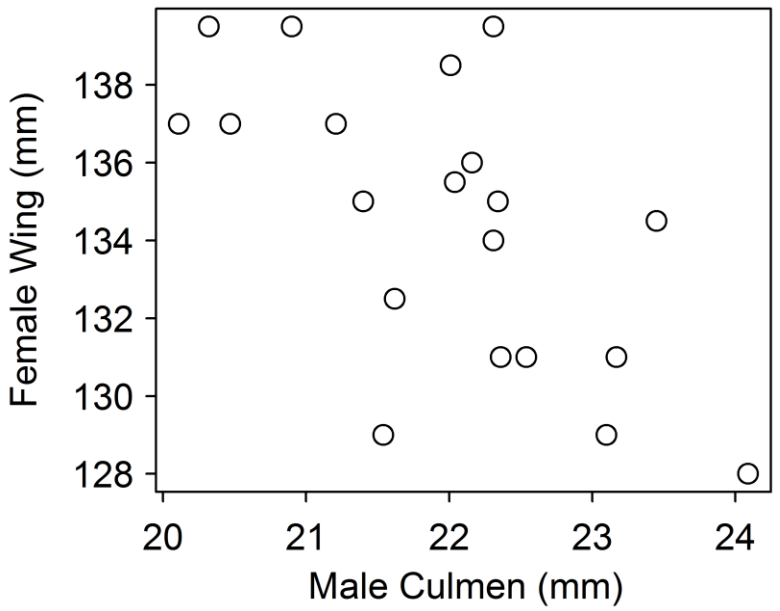


Fig. 7. The two most important traits explaining size assortative pairing were female wing and male culmen. Generally, males with larger beaks were paired with longer winged females, and to a lesser extent, females that were lighter in terms of mass (not shown).

Table 15. Loadings from the first axis of a PCA using size measures of male incubators. All traits contribute appreciably to the variation in this axis. PC1 explained 42% of the variation in size measures.

	PC1
Wing	0.420
Tarsus	0.617
Culmen	0.539
Mass	0.389

Male Incubator Size and Egg Size

I further investigated the relationship among mated pairs using the size of the incubating males and the eggs that they were incubating on the assumption that female size should be reflected in egg size. Because male culmen was identified as the prominent trait in the relationship with the size of the female mate, I did the analysis using both male culmen size as well as overall male size. Males (n=30) with smaller culmens were incubating eggs of greater volume (partial r=-0.41, P=0.02; year: $r_{partial}$=0.37, P=0.04; Fig. 8) and that were on average longer ($r_{partial}$=-0.44, P=0.01; year: $r_{partial}$=0.37, P=0.04). There was no relationship with egg width ($r_{partial}$=-0.23, P=0.22; year: $r_{partial}$=0.34, P=0.06). When using the first principal component representing overall body size (Table 15), the effects were similar but not as strong. Smaller males were incubating larger eggs (partial r=-0.35, P=0.05; year: $r_{partial}$=0.48, P=0.004). In a few cases, I found clutches of two (n=2) and three (n=7) eggs rather than four. I examined whether clutch size could be influencing average egg size, however there was little evidence that egg size and clutch size were related (clutch size vs average egg volume: $r_{spearman}$=-0.18, P=0.35; clutch size vs average egg length: $r_{spearman}$=-0.06, P=0.75; clutch size vs average egg width: $r_{spearman}$=-0.15, P=0.42).

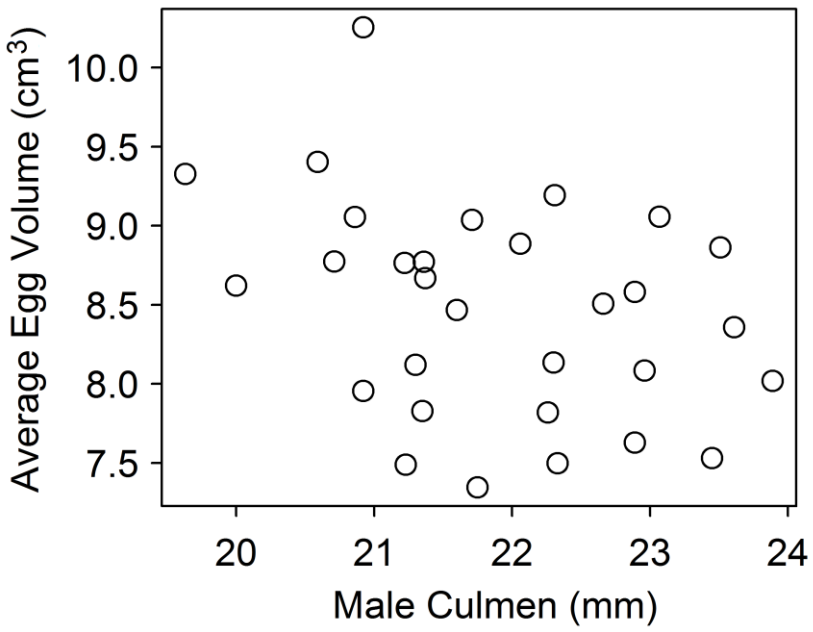


Fig. 8. Larger males, based on culmen size, were found to be incubating larger eggs. Male culmen was used to demonstrate this relationship specifically because it was identified as an important trait explaining pairing of adult birds.

Seasonal Date and Mate Choice Mechanisms

Including capture date in models had very little effect on the partial correlations among male and female traits (partial correlations with date included in models: male vs female breast PC2 with outlier included: $r_{partial}$=0.40, P=0.07; male culmen vs female wing: $r_{partial}$=-0.55, P=0.008; male culmen vs egg volume using estimated lay date: $r_{partial}$=-0.47, P=0.006). Further, date had little influence on any term across all analyses (absolute $r_{partial}$=0.02-0.33, P=0.94-0.29) except that eggs laid later in the season were larger (volume: $r_{partial}$=0.38, P=0.03). I also found no evidence that the strength of the relationship between paired male and female traits was heteroscedastic with respect to capture date (breast: BP=0.77, P=0.68; male culmen vs female wing: BP=2.12, P=0.35), nor was this the case for the relationship between male size and egg volume (BP=0.33, P=0.56).

Discussion

My findings show that Red phalaropes, despite being polyandrous and exhibiting a large degree of sexual size dimorphism and dichromatism, do indeed mate assortatively by both plumage colouration and body size. This demonstrates that traits of both sexes are sexually selected. Further, I found that pairing was not related to date, suggesting that assortative mating is not simply due to mate availability as a result of arrival time and the progression of the breeding season.

It has been well documented across a wide variety of organisms that more highly ornamented individuals are typically preferred by the opposite sex (Andersson 1994). In sexually dimorphic species, where only one sex possesses elaborate secondary sexual traits, mate choice is often thought to be absent in the sex that does not bear these traits; typically females (i.e., the 'choosy' sex). It is becoming clear though that mutual mate choice may occur under broader circumstances than previously thought, such as in sexually dimorphic species. Often 'non-choosy' males prefer large females (e.g., birds: Jönsson 1987; insects: Byrne and Rice 2006; crustaceans: Aquiloni and Gherardi 2008; fish: Paczolt and Jones 2010), where large female body size provides direct fecundity benefits for both sexes. Less common, and perhaps more interesting, are those species where the ornamented, 'non-choosy' sex prefers elaborated ornaments in the drab sex (Hill 1993, Amundsen *et al*. 1997, MacDougall and Montgomerie 2003, Pizzolon *et al*. 2008, Baldauf *et al*. 2011, Laczi *et al*. 2011).

Assortative Pairing based on Plumage Colour

As predicted, birds were paired positively based on breast plumage colour. The direction of the relationship is not surprising because it is well documented across a wide variety of organisms that more highly ornamented individuals are preferred by the opposite sex (Andersson 1994). In Chapter 5, I showed that breast plumage is condition-dependent in this population of phalaropes;

however, more colourful females had lower levels of plasma Immunoglobulin Y. In the present study, these more colourful females were paired with more colourful males. Consequently, it appears that mate choice for more colourful individuals does not achieve mating with individuals of higher immunological quality. On the other hand, more colourful individuals may be favoured if the negative relationship between plumage colour and immunity reflects a proximate trade-off with other beneficial aspects of reproduction, such as egg production.

Among the 20 pairs in this study, one pair consisted of a duller male and a colourful female and the inclusion of this point in the analysis obscured an otherwise strong relationship (Fig. 6). However, I might expect a certain amount of variability when measuring assortative mating in the wild because there are other constraints on the assessment of potential mates that means ideal mates are not always encountered. It is also worth noting that the pairing of a colourful female with a dull male is consistent with a relaxed preference in females which is the most likely scenario when facing limitations to mate assessment.

Body Size Relationship among Pairs and Incubating Males

Generally, females with longer wings (and less mass) were paired with males with smaller culmens. For female phalaropes, wing size may be particularly important because individuals with proportionally longer wings, especially if they also tend to be lighter, should have lower wing loading and greater agility; agility is an important trait given the aerial courtship displays used by shorebirds (Blomqvist *et al*. 1997, Figuerola 1999). Together, long wings and less mass reduces wing loading and should improve flight performance (Thaxter *et al*. 2010, Yalden 2012). Phalarope females are not territorial and do not perform typical aerial courtship displays, but they do undertake high speed, weaving chase flights which are an important component of courtship in phalaropes (Schamel and Tracy 1977, Tracy and Schamel 1988). It is not known

exactly how pairing outcomes are determined from chase flights, but it is likely that flight agility plays a role considering it is important for other shorebird species exhibiting aerial courtship displays (Blomqvist *et al*. 1997, Figuerola 1999). Phalaropes present an interesting situation though, because females are larger and presumably less manoeuvrable than males. If the outcome of aerial chases has tangible benefits for reproductive success, then there should be selection for agility among females. For female phalaropes, where large body size may be important for reducing the marginal costs of egg production (Liker *et al*. 2001), mate choice through chase flights could select for improved agility not through body size per se, but through wing loading. Indeed, this could explain why smaller males were mated to females specifically with larger wings.

Culmen size may also have a specific importance in mate choice. Sexual dimorphism in bill morphology has been linked to resource partitioning between males and females (Selander 1966, Temeles *et al*. 2000, Radford and du Plessis 2003). It has also been suggested that negative size assortative mating in dunlin (*Calidris alpina*), based on culmen length, could facilitate niche partitioning (Jönsson 1987). In Red phalaropes, feeding styles do appear to differ between the sexes, particularly so when males are actively incubating (Ridley 1980). Females more frequently feed below the surface of the water, often partially submerging in the process of catching prey. Males, on the other hand, tend to take food items directly from the water's surface, and selection on shorter beaks may facilitate this feeding style. Whereas sex differences in beak morphology have been thought to have an ecological origin (Székely *et al*. 2004), the present study, and others (e.g., Jönsson 1987), in fact suggest that mate choice could act on bill morphology that enhances niche partitioning.

Positive assortative mating with respect to size should result in a more appropriate matching of egg size of the incubating male. In doing so, it should reduce the costs of incubation for the male and presumably improve developmental conditions for the eggs (see Lislevand and Thomas 2006). Secondly, it should relax selection for small egg size in females if male-female size discrepancies are the root cause of small egg size in polyandrous species such as phalaropes (Lislevand and Thomas 2006). Contrary to my predictions, the results demonstrate the opposite pattern − small males are incubating large eggs − which suggests that mate choice does not function to reduce incubation costs, and may in fact place additional pressure on small egg size in females. Given the advantages of large eggs (Krist 2011), negative size assortative pairing in phalaropes seems advantageous for small males, at leats as measured by culmen size, and may therefore suggest small males are preferentially gaining access to large females.

My results show that in birds, mutual mate choice is not limited to species exhibiting mutual trait expression, nor to those where males and females have similar levels of parental care. To my knowledge, this is the first study of birds to demonstrate such a deviation from the basic theoretical predictions concerning mutual mate choice. In other organisms though, mutual mate choice has been observed in polygynous insects (Bonduriansky 2001, Bel-Venner *et al*. 2008) and polyandrous fish (Berglund *et al*. 2005). Generally, mutual mate choice can be expected to evolve when the benefits outweigh the costs (South and Arnqvist 2011). More specifically, theoretically unexpected mate choice can occur when high reproductive investment in the traditionally 'non-choosy' sex results in a decreased capacity to mate (Edward and Chapman 2011). Both of these views are suitable explanations for mutual pairing in phalaropes because 1) phalaropes are gregarious so that the cost of searching for a mate is probably quite low and the

opportunity to simultaneously assess multiple mates is high relative to other species, and 2) female phalaropes suffer a decreased capacity to mate, compared with an analogous polygynous male, because of the simultaneous costs of egg production.

Mating preferences place selection on the expression of phenotypic traits, and mutual mate choice/assortative mating does so in both sexes. Thus, the failure to detect these selection pressures underestimates the influence of sexual selection in the 'non-choosy' sex (Rosvall 2011). In phalaropes, where male plumage is dull compared to that of females, there is likely considerable selection for cryptic colouration in males because they incubate nests placed on the ground where they are vulnerable to predators. As a consequence, there is a conflict within males in which it is advantageous to be colourful, in terms of being preferred by females, while it is also advantageous to be dull, which reduces the likelihood for incubating males to be detected by predators while they incubate. The idea that there may be considerable conflict within the 'non-choosy' sex in terms of the expression of plumage clearly adds a new dimension to traditional views of sexual dichromatism driven by sexual conflict, where selection simply favours plumage exaggeration in one sex, and dull plumage in the other. The opposing selection pressures for colourful and dull plumage within males in phalaropes is certainly one avenue of future research. As such, I would expect the strength of mutual preferences to vary in predictable ways with changes in sex ratio and population size such that selection for male traits should be stronger when the sex ratio is more female-biased. This could be tested in phalaropes because they are known to undergo local fluctuations in population size (Tracy *et al*. 2002) which could influence the strength of sexual selection. Furthermore, I do not know how pairs form assortatively and what the consequences are for offspring quality and fitness, which would reveal the broader significance of assortative mating in this system.

Literature Cited

Amundsen, T. 2000. Why are female birds ornamented? Trends Ecol Evol 15: 149-155.

Amundsen, T., E. Forsgren and L.T.T. Hansen. 1997. On the function of female ornaments: male bluethroats prefer colourful females. Proc Biol Sci 264: 1579-1586.

Andersson, M. 1994. Sexual Selection. Princeton: Princeton University Press. 624p.

Aquiloni, L. and F. Gherardi. 2008. Mutual mate choice in crayfish: large body size is selected by both sexes, virginity by males only. J Zool 274: 171-179.

Baldauf, S.A., T.C.M. Bakker, H. Kullman and T. Thünken. 2011. Female nuptial coloration and its adaptive significance in a mutual mate choice system. Behav Ecol 22: 478-485.

Barry, K.L. and H. Kokko. 2010. Male mate choice: why sequential choice can make its evolution difficult. Anim Behav 80: 163-169.

Bel-Venner, M.C., S. Dray, D. Allainé, F. Menu and S. Venner. 2008. Unexpected male choosiness for mates in a spider. Proc Biol Sci 275: 77-82.

Berglund, A., M.S. Widemo and G. Rosenqvist. 2005. Sex-role reversal revisited: choosy females and ornamented, competitive males in a pipefish. Behav Ecol 16: 649-655.

Blomqvist, D. and O.C. Johansson. 1995. Trade-offs in nest site selection in coastal populations of Lapwings *Vanellus vanellus*. Ibis 137: 550-558.

Blomqvist, D., O.C. Johansson, U. Unger, M. Larsson and L.Å. Flodin. 1997. Male aerial display and reversed sexual size dimorphism in the dunlin. Anim Behav 54: 1291-1299.

Bonduriansky, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. Biol Rev Camb Philos Soc 76: 305-339.

Bortolotti, G.R., L.M. González, A. Margalida, R. Sánchez and J. Oria. 2008. Positive assortative pairing by plumage colour in Spanish imperial eagles. Behav Processes 78: 100-107.

Byrne, P.G. and W.R. Rice. 2006. Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. Proc Biol Sci 273: 917-922.

Edward, D.A. and T. Chapman. 2011. The evolution and significance of male mate choice. Trends Ecol Evol 26: 647-654.

Edwards, D.B., and H.G. Gilchrist. 2011. A new means of catching shorebirds: the Super Talon net gun. Wader Study Group Bulletin 118: 134-6.

Edwards, D.B., E.H. Chin, G. Burness, A.I. Schulte-Hostedde and H.G. Gilchrist. 2013. Linking sex differences in corticosterone with individual reproductive behaviour and hatch

success in two species of uniparental shorebirds. Comp Biochem Physiol A 166: 169-176.

Figuerola, J. 1999. A comparative study on the evolution of reversed size dimorphism in monogamous waders. Biol J Linn Soc Lond 67: 1-8.

González, I. and S. Déjean. 2009. CCA: Canonical correlation analysis. R package version 1.2. http://CRAN.R-project.org/package=CCA.

Göth, A., and C.S. Evans. 2004. Egg size predicts motor performance and postnatal weight gain in Australian Brush-turkey (*Alectura lathami*) hatchlings. Can J Zool 82: 972-979.

Grant, M.C. 1991. Relationships between egg size, chick size at hatching, and chick survival in the Whimbrel *Numenius phaeopus*. Ibis 133: 127-133.

Griggio, M., A. Devigili, H. Hoi and A. Pilastro. 2009. Female ornamentation and directional male mate preference in the rock sparrow. Behav Ecol 20: 1072-1078.

Hair, J.F., W.C. Black, B.J. Babin and R.E. Anderson. 2009. Multivariate Data Analysis, 7th ed. Upper Saddle River: Prentice Hall, 2009.

Hill, G.E. 1993. Male mate choice and the evolution of female plumage coloration in the house finch. Evolution 47: 1515-1525.

Howe, M.A. 1975. Social interactions in flocks of courting Wilson's phalaropes (*Phalaropus tricolor*). Condor 77: 24-33.

Johns, J.E. 1964. Testosterone-induced nuptial feathers in phalaropes. Condor 66: 449-455.

Johnstone, R.A., J.D. Reynolds and J.C. Deutsch. 1996. Mutual mate choice and sex differences in choosiness. Evolution 50: 1382-1391.

Jones, A.G. and N.L. Ratterman. 2009. Mate choice and sexual selection: What have we learned since Darwin? Proc Natl Acad Sci USA 106: 10001-10008.

Jönsson, P.E. 1987. Sexual size dimorphism and disassortative mating in the Dunlin *Calidris alpina schinzii* in Southern Sweden. Ornis Scandinavica 18: 257-264.

Kim, S. 2012. ppcor: Partial and Semi-partial (Part) correlation. R package version 1.0. http://CRAN.R-project.org/package=ppcor.

Kimball, R.T., and J.D. Ligon. 1999. Evolution of avian plumage dichromatism from a proximate perspective. Am Nat 154: 182-193.

Kokko, H., and R.A. Johnstone. 2002. Why is mutual mate choice not the norm? Operational sex ratios, sex roles and the evolution of sexually dimorphic and monomorphic signalling. Proc Biol Sci 357: 319-330.

Kraaijeveld, K., F.J.L. Kraaijeveld-Smit and J. Komdeur. 2007. The evolution of mutual ornamentation. Anim Behav 74: 657-677.

Krist, M. 2011. Egg size and offspring quality: a meta-analysis in birds. Biol Rev Camb Philos Soc 86: 692-716.

Laczi, M, J. Török, B. Rosivall and G. Hegyi. 2011. Integration of spectral reflectance across the plumage: implications for mating patterns. PLoS ONE 6: e23201.

Ledwoń, M. 2011. Sexual size dimorphism, assortative mating and sex identification in the Whiskered Tern Chlidonies hybrid. Ardea 99: 191-198.

Liebezeit, J.R., P.A. Smith, R.B. Lanctot, H. Schekkerman, I. Tulp, S.J. Kendall, D.M. Tracy, R.J. Rodrigues, H. Meltofte, J.A. Robinson, C. Gratto-Trevor, B.J. McCaffery, J. Morse, and S.W. Zack. 2007. Assessing the development of shorebird eggs using the flotation method: Species-specific and generalized regression models. Condor 109: 32-47.

Liker, A., J.D. Reynolds and T. Székely. 2001. The evolution of egg size in socially polyandrous shorebirds. Oikos 95: 3–14.

Lislevand, T. and G.H. Thomas. 2006. Limited male incubation ability and the evolution of egg size in shorebirds. Biol Lett **2**: 206-208.

MacDougall, A.K. and R. Montgomerie. 2003. Assortative mating by carotenoid-based plumage colour: a quality indicator in American goldfinches, *Carduelis tristis*. Naturwissenschaften 90: 464-467.

McGraw, K.J. 2006. The mechanics of melanin-based coloration. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 243–294.

Menzel, U. 2011. CCP: Significance Tests for Canonical Correlation Analysis (CCA). R package version 1.1. http://CRAN.R-project.org/package=CCP.

Møller, A.P. 1994. Phenotype-dependent arrival time and its consequences in a migratory bird. Behav Ecol Sociobiol 35: 115-122.

Narushin, V.G. 2005. Egg geometry calculation using the measurements of length and breadth. Poult Sci 84: 482- 484.

Nol, E., A.J. Baker and M. D. Cadman. 1984. Clutch initiation dates, clutch size, and egg size of the American Oystercatcher in Virginia. Auk 101: 855-867.

Nol, E., MS.S. Blanken and L. Flynn. 1997. Sources of variation in clutch size, egg size and clutch completion dates of Semipalmated Plovers in Churchill, Manitoba. Condor 99: 389-396.

Paczolt, K.A. and A.G. Jones. 2010. Post-copulatory sexual selection and sexual conflict in the evolution of male pregnancy. Nature 464: 401-404.

Pizzolon, M., M.B. Rasotto and C. Mazzoldi. 2008. Male lagoon gobies, *Knipowitschia panizzae*, prefer more ornamented to larger females. Behav Ecol Sociobiol 62: 521-528.

Prum, K.J. 2006. Anatomy, physics, and evolution of structural colors. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 295–353.

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Radford, A.N. and M.A. du Plessis. 2003. Bill dimorphism and foraging niche partitioning in the green woodhoopoe. J Anim Ecol 72: 258-269.

Redmond, R. L. 1986. Egg size and laying date of Long-billed Curlews *Numenius americanus*: implications for female reproductive tactics. Oikos 46: 330-338.

Ridley, M.W. 1980. The breeding behavior and feeding ecology of Grey Phalaropes *Phalaropus fulicarius* in Svalbard. Ibis 122: 210-226.

Rosvall, K.A. 2011. Intrasexual competition in females: evidence for sexual selection? Behav Ecol 22: 1131-1140.

Rowe, K.M.C. and P.J. Weatherhead. 2011. Assortative mating in relation to plumage traits shared by male and female American robins. Condor 113: 881-889.

Schamel, D., and D. Tracy. 1977. Polyandry, replacement clutches, and site tenacity in the Red Phalarope (*Phalaropus fulicarius*) at Barrow, Alaska. Bird-Banding 48: 314–324.

Schamel, D., D.M. Tracy and D.B. Lank. 2004. Male mate choice, male availability and egg production as limitations on polyandry in the red-necked phalarope. Anim Behav 67: 847-853.

Selander, R.K. 1966. Sexual dimorphism and differential niche utilization in birds. Condor 68: 113-151.

Silva, N., J.M. Avilés, E. Danchin and D. Parejo. 2008. Informative content of multiple plumage-coloured traits in female and male European Rollers. Behav Ecol Sociobiol 62: 1969-1979.

South, S.H. and G. Arnqvist. 2011. Male but not female, preference for an ornament expressed in both sexes of the polygynous mosquito *Sabethes cyaneus*. Anim Behav 81: 645-651.

Székely, T., R.P. Freckleton and J.D. Reynolds. 2004. Sexual selection explains Rensch's rule of size dimorphism in shorebirds. Proc Natl Acad Sci USA 101: 12224-12227.

Temeles, E.J., I.L. Pan, J.L. Brennan and J.N. Horwitt. 2000. Evidence for ecological causation of sexual dimorphism in a hummingbird. Science 289: 441-443.

Thaxter, C.B., S. Wanless. F. Daunt, M.P. Harris, S. Benvenuti, Y. Watanuki, D. Gremillet and K.C. Hamer. 2010. Influence of wing loading on the trade-off between pursuit-diving and flight in common guillemots and razorbills. J Exp Biol 213: 1018-1025.

Tracy, D., and D. Schamel. 1988. Male initiation of pair formation in Red Phalaropes. Wilson Bulletin 100: 144-147.

Tracy, D.M., D. Schamel, and J. Dale. 2002. Red Phalarope (*Phalaropus fulicarius*). *The Birds of North America Online* (A. Poole, Ed.). Cornell Lab of Ornithology: Ithaca, NY.

Whitfield, D. P. 1995. Behaviour and ecology of a polyandrous population of Grey Phalaropes *Phalaropus fulicarius* in Iceland. J Avian Biol 26: 349–352.

Yalden, D.W. 2012. Wing area, wing growth and wing loading of Common Sandpipers *Actitis hypoleucos.* Wader Study Group Bulletin 119: 84–88.

Zeileis, A. and T. Hothorn. 2002. Diagnostic Checking in Regression Relationships. R News 2: 7-10. URL http://CRAN.R-project.org/doc/Rnews/.

CHAPTER 7: General Conclusion

The predominant focus of this thesis has been to examine the relationship between reproduction and immune function, as considered from the perspectives of life history theory and signalling theory (i.e., the Immunocompetence Handicap Hypothesis, ICHH). To achieve the outcomes of the thesis, I used two model biological systems: Psittaciformes and Charadriiformes. Psittaciformes (parrots) are amenable to study immune investment for several reasons. Firstly, they are generally long lived and, because they are commonly kept in captivity, there are published accounts of lifespan potential for a variety of species. Measures such as these are not as readily available for other study systems. Secondly, parrots are largely sexually monochromatic which should limit confounding intrasexual selection for plumage colouration that could constrain the expression of colour as a result of mate choice. The final reason is comparative studies are largely focussed on Passeriformes (songbirds), and so using another group broadens our understanding by increasing the phylogenetic scope of studies.

The remainder of the thesis is focussed on the Charadriiformes, and phalaropes in particular. This is the first study to look at immune investment in a sex-role reversed bird, despite the potential for such systems to inform on the general biological concepts developed using conventional systems (Forbes 2007, Hasselquist 2007). The first work to be published on sex differences in immunity in a role reversed vertebrate occurred only during the course of my thesis (pipefish: Roth *et al*. 2011). My work in this thesis differs by having a focus on the hormones that are often invoked to explain sex differences in immunity, chiefly testosterone.

Male-biased immunosuppression is thought to be the result of testosterone, and may be the result of energetic-based trade-offs between reproduction and survival, or as a handicap maintaining the honest expression of secondary sexual traits; the Immunocompetence Handicap Hypothesis (ICHH). Rarely are these two concepts treated simultaneously. Although there has

been overwhelming acceptance of the ICHH, the simple relationship between testosterone and immunity is debated (Roberts *et al*. 2004).

The ICHH has suffered from a lack of clear expectations, particularly in terms of how the costs of plumage expression are borne across individuals. On one hand, plumage expression should be costly and more ornamented individuals should suffer a greater handicap; that is the degree of plumage expression should be negatively related to immunity. On the other hand, more highly ornamented individuals may be better able to pay the costs of immunosuppression. Because of the unclear predictions, both positive and negative relationships have been offered as support for the ICHH (Braude *et al*. 1999). However, the variability in these relationships suggests that a functionally singular ICHH mechanism is not present: in some cases it can be positive and in others negative, and that the nature of the results may be determined by species differences or environmental conditions.

That brighter species have more parasites (Hamilton and Zuk 1982) was an important observation leading to the ICHH by which it was suggested that these species were handicapped by the elaborate expression of their traits (Folstad and Karter 1992). Subsequently, it has become evident that this is not the case, even though relatively little comparative work has been done in follow up to Hamilton and Zuk (1982). Some studies have revealed a positive relationship between the degree of sexual dichromatism and immune investment across species (Møller 1997, Møller *et al*. 1998) demonstrating that immunity and aspects of plumage ornamentation are positively related. More directly, in Chapter 2 I demonstrated that more brightly coloured parrot species have higher immune investment. However, I found that dichromatism was negatively related to immunity. Together, these results are not consistent with general costs of exaggerated plumage traits, particularly in terms of immunosuppression. The inconsistency is either that

species can overcome these costs or, more likely, that mate choice works to increase investment in both secondary sexual traits and immunity.

The observation that sex differences in immunity often occur was an important observation for the ICHH (Folstad and Karter 1992), which implicated testosterone in the regulation of both immunity and the expression of sexual signals, resulting in immunosuppression among males. Subsequently, we have come to understand that reproductive decisions, as well as the regulation of immune function, is complex and likely controlled by a number of hormones. Corticosterone has received a lot of attention in this regard as it has well known effects on the immune system and behaviour (Wingfield *et al*. 1998, Sapolsky *et al*. 2000). Sex differences in corticosterone could produce sex differences in immunity because corticosterone often suppresses immune function (Råberg *et al*. 1998).

In birds, sex differences in corticosterone are frequently observed, the direction of which appears to be related to mating system/parental sex roles (O'Reilly and Wingfield 2001). O'Reilly and Wingfield (2001) posited that incubating birds benefit from tolerating stressors that could cause them to abandon their clutch, which is why the incubating sex appears to have lower levels of stress-induced corticosterone than the non-incubating sex. This mechanism, the reduction of stress-induced corticosterone during incubation creating sex differences, opposes the notion that courtship and agonistic interactions are stressful and energetically costly (Barnett and Briskie 2007, Greives *et al*. 2007, Lynn *et al*. 2010) which could increase stress levels in the non-incubating sex that competes for mates. In Chapter 3, I addressed the idea that corticosterone is reduced in incubating birds by linking both baseline and stress-induced corticosterone levels to individual nest defence behaviour and hatch success in polygynous and polyandrous species of shorebirds. I found only partial support for this notion in Red phalaropes (where only males

incubate) and little support for it in White-rumped sandpipers (where only females incubate). Moreover, I found that sex differences in corticosterone were present prior to the incubation period in phalaropes, demonstrating that sex differences in corticosterone are the result of broader sex-specific reproductive functions, not just incubation. With that said, although sex differences were present when males and females were in the courtship period, corticosterone levels were lower in males during incubation than males during courtship.

Over the years, the ICHH has been expanded to include a role for corticosterone (Møller 1995, Owen-Ashley *et al*. 2004, Roberts *et al*. 2007). In Chapter 4, I examined how testosterone and corticosterone contributed to sex-biased immunocompetence in Red phalaropes. Female phalaropes had poorer immunocompetence relative to males supporting the pattern that the sex competing for mates (typically males) is immunosuppressed, and which reinforces the notion of a Bateman's-like trade-off. Females had higher levels of corticosterone and lower levels of some measures of the immune system; males had higher testosterone [and were not immunosuppressed relative to females]. On one hand, this suggests that corticosterone but not testosterone contributes to sex-biased immunocompetence. On the other hand, testosterone appears to be immunomodulating at the level of the individual because females with high levels of testosterone had fewer plasma immunoglobulins, but more leukocytes. Of importance is that these actions were sex-specific, and they were not dose-dependent because testosterone levels were not related to immune measures in males.

An ICHH-like handicap is supposed to mediate honesty (Folstad and Karter 1992). As such, traits being mediated by such a mechanism should be reliable indicators of individual quality (addressed in Chapter 5) that are then used in mate choice (addressed in Chapter 6). Indeed, aspects of plumage colouration were condition-dependent in both males and females

suggesting that a mechanism could exist to maintain 'honesty' in the traits of both males and females. The specific mechanism cannot be addressed in this thesis but in Chapter 4 I found no evidence that immunity was regulated by testosterone in males, or, in Chapter 5, that plumage colouration was related to testosterone in males. As a consequence, there does not seem to be a common mechanism in both males and females to ensure reliability of signals.

A direct interpretation of an immunocompetence handicap is that higher levels of testosterone result in greater immunosuppression. Because higher levels of testosterone are responsible for more colourful traits, the implication is that more colourful individuals should be more immunosuppressed. Although this is one expectation, it is rarely found (e.g., Faivre *et al*. 2003, Aguilera and Amat 2007, Vinkler *et al*. 2011). However, this is what I found in Chapter 5: colouration and immunity were negatively related, which supports the simplest form of the 'handicap mechanism'. That the most colourful individuals were in poorer immunological condition is not commonly seen and begs the question as to why in some species, or under some conditions, this is the case. This has yet to be studied in detail.

Although the results from Chapter 5 seem counter-intuitive, phalaropes do pair based on plumage colouration, with more colourful individuals pairing with each other. Independently of colouration, phalaropes also mate based on size. Taken together, the results from Chapters 4, 5 & 6 demonstrate that sex-role reversed phalaropes use condition-dependent signals when selecting mates, and may be immunosuppressed as a consequence of bearing those traits.

The findings of Chapter 5 & 6 also provide insight into the types of traits that are used in signalling and mate choice. The reddish breast plumage and the black crown plumage are produced by the pigment melanin. More specifically, eumelanin is the most common form of melanin and chiefly produces dark brown and black plumage (McGraw 2006a), as in the crown

plumage of phalaropes. Pheomelanin is responsible for reddish-orange plumage (McGraw 2006a), as in the breast plumage of phalaropes. Alternatively, the white plumage is caused by light reflecting off the microstructure of the feather (Prum 2006). Although I found evidence that these plumage types were condition-dependent and/or used in mate choice, the strongest relationships involved the breast plumage (pheomelanin). Interestingly, the metabolic role of pheomelanin is not as clear as it is for other pigments used in signalling (see Galván and Møller 2013), such as eumelanin (McGraw 2006a) or carotenoids (McGraw 2006b). Knowledge of the metabolic function of pheomelanin could clarify its role in signalling. For example, because carotenoids are antioxidants their deposition into feathers could reflect the individual's ability to buffer damage from free radicals (von Schantz *et al*. 1999). Interestingly, the black crown plumage, one of the most sexually dimorphic plumage traits in Red phalaropes, was only weakly condition-dependent and did not explain patterns in pairing. This supports the general notion that eumelanin-based plumage traits such as these are not often used as signals of individual quality in mate choice (McGraw 2008).

Feather hue was by far the most important of the spectral measures contributing to both condition-dependence and mate choice; hue was important for most traits but more so for the pheomelanin of breast plumage. This is consistent with the expectation that hue should be the most likely spectral component to resolve condition dependence because of the direct relationship between values of hue and the amount of pigment deposited into feathers (Andersson 1999).

As a result of this thesis, I have provided some support for the central tenets of the ICHH, but also evidence against the handicap process. At the fundamental core of the ICHH, is the observation that testosterone can be responsible for the production of sexual signals used in mate

choice as well as immunosuppression, and at face value the phalarope work demonstrates these concepts. However, in Chapter 2 I demonstrated that there is not an overt cost to immunocompetence in expressing elaborate plumage traits because species with more elaborate traits had higher investment in immunocompetence. Furthermore, I demonstrated that males can circumvent the costs of high testosterone because female phalaropes had poorer immunocompetence despite high levels of testosterone in males. Although the lack of a relationship between testosterone and plumage colouration in Chapter 5 may result from the bimodality of testosterone titres in males, the results from this chapter also demonstrate that males must achieve condition-dependent expression of traits based on different levels of testosterone than females.

There are two prominent themes in this thesis: life history trade-offs involving immunity, and the Immunocompetence Handicap Hypothesis. Many, including myself, consider these concepts separate processes (e.g., Rolff 2002, Nunn *et al*. 2009). However, direct tests to distinguish between them are at the very least difficult, and some have considered the ICHH altogether untestable (Oliveira 2004). Throughout much of this thesis I have used language that reflects the contemporary views of the ICHH. In the conclusion, I have chosen to express my skepticism of biological handicaps maintaining honesty in plumage traits via an 'immunocompetence handicap' by adopting single quotes around terms such as 'handicap', or by replacing loaded terms, such as 'honesty', with more preferable terms, such as 'reliability'. Whereas the ICHH originated as a mechanistic explanation for signal honesty maintained through a biological handicap, the current usage of the ICHH may refer to both a biological handicap, as well as the simple observations between testosterone, signal production and

immunocompetence. To speak to these points, I have amalgamated my thoughts on the ICHH as a handicap mechanism in Appendix 3 in order to complement the results of this thesis.

Literature Cited

Andersson, S. 1999. Efficacy and content in avian signals. In: Espmark, Y., T. Amundsen and G. Rosenqvist (Eds), Adaptive Signals Signalling and Signal Design in Animal Communication. Tapir Publishers, Trondheim, (NO). pp. 47-60.

Aguilera, E. and J.A. Amat. 2007. Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). Naturwissenschaften 94: 895-902.

Barnett, C.A. and J.V. Briskie. 2007. Energetic state and the performance of dawn chorus in silvereyes (*Zosterops lateralis*). Behav Ecol Sociobiol 61: 579-587.

Braude, S., Z. Tang-Martinez and G.T. Taylor. 1999. Stress, testosterone, and the immunoredistribution hypothesis. Behav Ecol 10: 345-350.

Folstad, I., and A.J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603-622.

Faivre, B., M. Préault, F. Salvadori, M. Théry, M. Gaillard and F. Cézilly. 2003. Bill colour and immunocompetence in the European blackbird. Animal Behaviour 65: 1125‑1131.

Forbes, M.R. 2007. On sex differences in optimal immunity. Trends Ecol Evol 22:111-113.

Galván, I. and A.P. Møller. 2013. Pheomelanin-based plumage coloration predicts survival rates in birds. Physiol Biochem Zool 86: 184-192.

Greives, T.J., J.M. Casto and E.D. Ketterson. 2007. Relative abundance of males to females affects behaviour, condition and immune function in a captive population of dark-eyed juncos *Junco hyemalis*. J Avian Biol 38: 255-260.

Hamilton, W.D., and M. Zuk. 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Hasselquist, D. 2007. Comparative immunoecology in birds: hypotheses and tests. J Ornithol 148: S571-S582.

Lynn, S.E., T.B. Stamplis, W.T. Barrington, N. Weida and C.A. Hudak. 2010. Food, stress, and reproduction: Short-term fasting alters endocrine physiology and reproductive behaviour in the zebra finch. Horm Behav 58: 214-222.

McGraw, K.J. 2006a. The mechanics of melanin-based coloration. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 243–294.

McGraw, K.J. 2006b. The mechanics of carotenoid-based coloration. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 177–242.

McGraw, K.J. 2008. An update on the honesty of melanin-based color signals in birds. Pigment Cell Melanoma Res 21: 133-138.

Møller AP. 1995. Hormones, handicaps and bright birds. Trends Ecol Evol 10:121.

Møller, A.P. 1997. Immune defence, extra-pair paternity, and sexual selection. Proc Biol Sci 264: 561-566.

Møller, A.P., R. Dufva and J. Erritzøe. 1998. Host immune function and sexual selection in birds. J Evol Biol 11: 703-719.

Nunn, C.L., P. Lindenfors, E.R. Pursall and J. Rolff. 2009. On sexual dimorphism in immune function. Phil Trans R Soc B 364: 61-69.

Oliveira, R.F. 2004. Social modulation of androgens in vertebrates: mechanisms and function. Adv Study Behav 34: 165-239.

O'Reilly, K.M. and J.C. Wingfield. 2001. Ecological factors underlying the adrenocortical response to capture stress in arctic-breeding shorebirds. Gen Comp Endocrinol 124: 1-11.

Owen-Ashley, N.T., D. Hasselquist, and J.C Wingfield. 2004. Androgens and the immunocompetence handicap hypothesis: Unraveling direct and indirect pathways of immunosuppression in Song Sparrows. Am Nat 164: 490-505.

Prum, K.J. 2006. Anatomy, physics, and evolution of structural colors. In: Hill, G.E. and K.J. McGraw (Eds), Bird Coloration. 1. Mechanisms and Measurements. Harvard University Press, Cambridge, (MA). pp. 295–353.

Råberg, L., M. Grahn, D. Hasselquist, and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. Proc Biol Sci 265: 1637-1641.

Roberts, M.L., K.L. Buchanan, and M.R. Evans. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim Behav 68: 227-239.

Roberts, M.L., K.L. Buchanan, D. Hasselquist, and M.R. Evans. 2007. Effects of testosterone and corticosterone on immunocompetence in the zebra finch. Horm Behav 51: 126-134.

Rolff, J. 2002. Bateman's principle and immunity. Proc Biol Sci 269: 867-872.

Roth, O., J.P. Scharsack, I. Keller and T.B.H. Reusch. 2011. Bateman's principle and immunity in a sex-role reversed pipefish. J Evol Biol 24: 1410-1420.

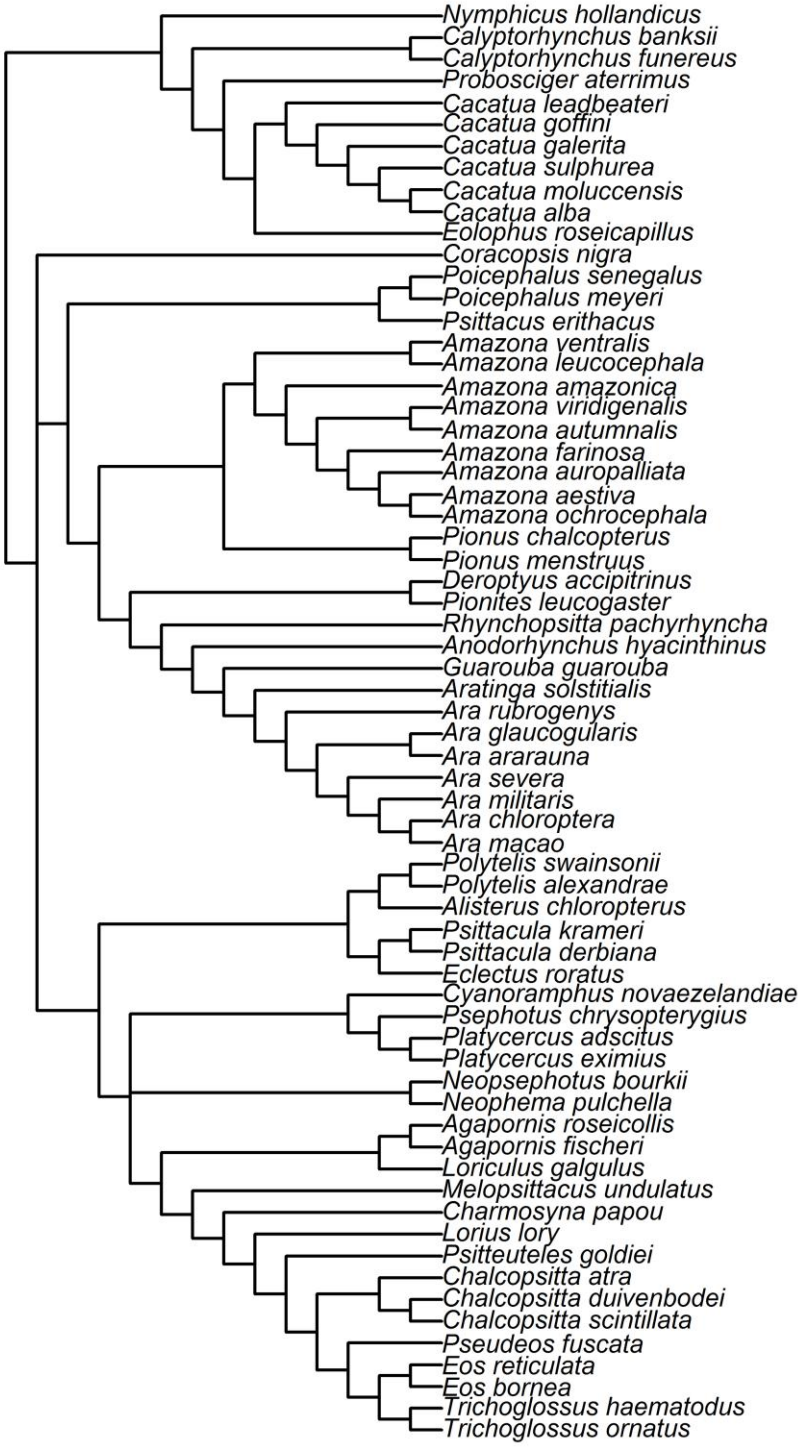
Sapolsky, R.M., M. Romero, and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparatory actions. Endocr Rev 21: 55-89.

Vinkler, M., J. Schnitzer, P. Munclinger and T. Albrecht. 2011. Phytohaemaglutinin skin-swelling test in scarlet rosefinch males: low-quality birds respond more strongly. Anim Behav 83: 17-23.

von Schantz, T., S. Bensch, M. Grahn, D. Hasselquist and H. Wittzell. 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B 266: 1–12.

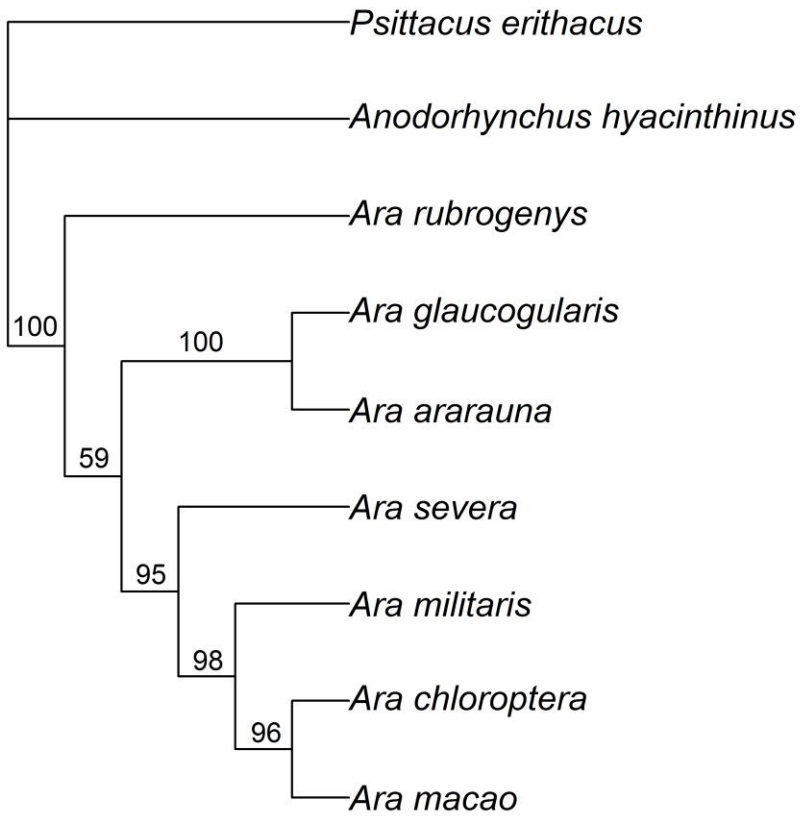
Wingfield, J.C., D.L. Maney, C.W. Breuner, J.D. Jacobs, S. Lynn, M. Ramenofsky and R.D. Richardson. 1998. Ecological bases of hormone-behavior interactions: the "emergency life history stage". Am Zool 38: 191-206.

Appendix 1: Parrot Phylogeny



Appendix 1. Phylogeny of the 66 parrot species from Chapter 2 for which there were leukocyte data. The tree is that of Mayr (2010) with modifications from several other sources (see Chap2: Methods).

Appendix 2: Phylogeny for the *Ara* genus.



Appendix 2. Bayesian tree constructed from a 430bp RNA 16s gene fragment for the genus *Ara*. Posterior probabilities evidence strong support for most branches in the tree. *P. erithacus* and *A. hyacinthinus* were used as outgroups.

APPENDIX 3: Handicapped Honesty or Natural Reliability? Revisiting the Immunocompetence Handicap Hypothesis 20 Years Later.

The ICHH has been extremely influential. At the time of writing, Folstad and Karter (1992) had garnered 1,286 citations (Fig. 1); it is one of the most highly cited papers in ecology and evolution published in the past 25 years. The work simultaneously brought to the forefront of ecology and evolution the notion that the immune system is an important component of sexual signals, and that testosterone could be immunosuppressive. Both of these concepts have subsequently been linked to life history theory in a larger context.

What is the ICHH?

In its simplest form, the ICHH is based on the "double-edged sword" effect of testosterone: that testosterone is responsible for the production and degree of expression of secondary sexual traits, and that testosterone is immunosuppressive (Folstad and Karter 1992). Explicitly, the ICHH provides a mechanism to maintain honesty of signals by imposing a handicap on the bearer of elaborate sexual ornaments, a handicap that is facilitated by hormonal regulation of immune function.

The handicap phenomenon is based on the Zahavi handicap principle (Zahavi 1975). The idea has subsequently been supported by models suggesting that signals require a handicap in order to maintain their honesty (Grafen 1990, Maynard Smith 1991). Rather than inherent production costs, handicaps are additional, wasteful costs required to maintain the honest expression of a signal where a conflict of interest between the signaller and receiver exists (Zahavi and Zahavi 1997, Számadó 2011).

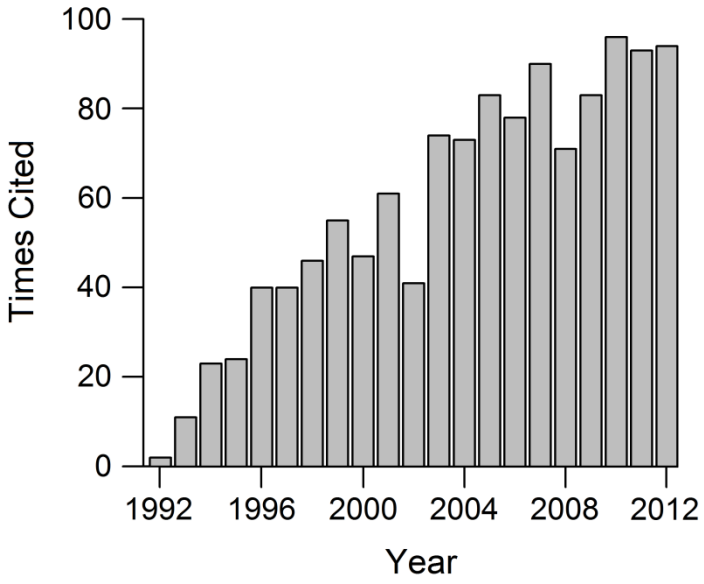


Fig.1A. The citation record for the Immunocompetence Handicap Hypothesis (Folstad and Karter 1992) based on citations recorded by Web of Science. The number of citations has not been corrected for an overall increase in publication rate since 1992, but clearly the ICHH continues to garner considerable attention.

'Honesty' is a term that is intrinsically associated with handicaps. The implication of the ICHH is that cheating can occur without the hormonal handicap. This fact is recognized as a fundamental difference between handicap mechanisms and intrinsic indices of quality (e.g., Shingleton and Frankino 2012). Folstad and Karter (1992) state the handicap mechanism explains signalling phenomena beyond what can be explained by energetic evidence; the authors claim that testosterone dependent "maladaptive immunosuppression" producing an honest signal is in addition to other processes, such as basic production costs. Indeed, many authors recognize handicaps, and specifically the ICHH, to be separate from other processes that could maintain reliable signals or explain phenomena related to immune expression. Rolff (2002), and more recently Nunn *et al*. (2009), argue that sex-biased immunosuppression can be explained by Bateman's Principle, and contrast this directly with the ICHH. Poiani *et al*. (2000) assert that the

ICHH plays on important role in maintaining honesty, *but should it fail to do so*, cheating can be eliminated through natural selection. Thus, in the spirit of signalling handicaps, the ICHH is considered separate to processes such as natural selection, and opposes the notion of inherent reliability.

Although the idea has been widely embraced, a number of criticisms have been raised and the current status of the ICHH is still questionable. A major criticism is that the ICHH does not make clear predictions and therefore is not testable (Braude *et al*. 1999, Oliveira 2004). Below, I outline several additional criticisms in detail.

Testosterone-mediated Immunosuppression as a Critical Component of the ICHH

The main focus of the ICHH debate has been on whether testosterone is in fact immunosuppressive. At the centre, is a meta-analysis that concluded there is little evidence of testosterone-mediated immunosuppression in experimental studies (Roberts *et al*. 2004). This study forms the catalyst for arguments against the ICHH. Studies of birds have provided both support for testosterone-mediated immunosuppression (Deviche and Cortez 2005, Edler *et al*. 2011) and against it (Hasselquist *et al*. 1999, Evans *et al*. 2000, Roberts *et al*. 2007, Roberts *et al*. 2009), and it would be interesting to revisit the argument using a meta-analytical approach using more recent studies while asking how phylogeny, immunological assay, and species life history traits influence these outcomes.

Whereas the question of whether testosterone *does* suppress immune function is still unanswered, or perhaps is answered due to underwhelming support, the answer to the question of whether it *can* is clearly yes. The observation that in many animals lymphocytes possess

androgen receptors (salmonid fish: Slater *et al*. 1995, mice: Liva and Voskuhl 2001) is proof that testosterone, or its derivatives, play some role in modulating the immune response directly. To my knowledge, androgen receptors have not explicitly been identified on avian leukocytes. However, phagocytic activity of avian macrophages is suppressed in the presence of testosterone *in vitro* (Gil and Culver 2011) providing strong evidence of the existence of a direct action of androgens on macrophages, but nevertheless demonstrates that testosterone can interact directly with immune system components to influence their activity. Moreover, testosterone can influence the susceptibility to parasites irrespective of behaviours that accompany testosterone (Mougeot *et al*. 2005), again demonstrating a direct role for testosterone. Testosterone-mediated immunosuppression has received more attention than other aspects of the ICHH and my aim here is to merely highlight aspects to give the essence of the current state of knowledge. What is apparent is that studies demonstrating immunosuppression related to testosterone levels are not uncommon suggesting that, under certain circumstances, testosterone can be mmunosuppressive.

There are numerous indirect pathways by which testosterone can alternatively modulate immunity. In birds, testosterone appears to have a relationship with corticosterone. Corticosterone increases with testosterone levels seasonally (Romero and Wingfield 1998, Breuner and Orchinik 2001) as well as under short-term experimental conditions (Poiani *et al*. 2000, Casto *et al*. 2001, Mateos 2005, Ashley *et al*. 2009). Because corticosterone is immunosuppressive (Råberg *et al*. 1998, Buchanan 2000, Sapolsky *et al*. 2000), corticosterone-mediated immunosuppression could be a side-effect of elevated levels of testosterone. This mechanism is a potentially important amendment to the ICHH (Owen-Ashley *et al*. 2004).

Androgen hormones structurally related to, and in many cases derived from, testosterone may also be an indirect mode of action of testosterone. Although not tested directly in birds to

my knowledge, dihydrotestosterone can suppress important cytokines such as interleukin (IL)-4, IL-5 and interferon in lab mice (Araneo *et al*. 1991). Cytokines generally improve immune system functioning via several mechanisms including proliferation and activation of immune cells, increasing antigen presentation to lymphocytes and MHC regulation (Young and Hardy 1995, Shtrichman and Samuel 2001, Okado *et al*. 2003, Takatsu *et al*. 2009). To date, an indirect role involving dihydrotestosterone in birds is not well supported though (Owen-Ashley *et al*. 2004).

Testosterone functions heavily in the original hypothesized ICHH mechanism. Indeed, the actions of testosterone are so fundamental to the popular interpretation of the ICHH mechanism that support for the ICHH seemingly hinges upon evidence for testosterone-mediated immunosuppression. As such, invertebrates are a model that are often invoked as evidence against the ICHH because males possess elaborate sexual traits and are immunosuppressed relative to females but they do not have sex specific hormones (e.g., Nunn *et al*. 2009). Whereas the independent evolution of a functionally similar but physiologically different handicap mechanism may not be parsimonious, the lack of testosterone does not disprove the ICHH. Folstad and Karter (1992) themselves assert that "any biochemical structure" that is responsible for trait expression as well as immunosuppression could fill the handicap role. In insects, juvenile hormone could fill the role as a mediator of a handicap because it produces sexual dimorphisms (Shelby *et al*. 2007) and is immunosuppressive (Rantala *et al*. 2003).

Evidence against the ICHH

Condition Dependence of Testosterone

Sexual signals convey information about their bearers. Although some signals are considered badges of status (Senar 2006), more often the information conveyed is that of the condition of the individual. Thus, sexual signals are usually considered condition-dependent. Cheaters then misrepresent their condition by producing a more elaborate signal which makes them appear to be a superior mate. Testosterone purportedly prevents cheating through immunosuppression: described in the ICHH. However, testosterone has been shown to be condition-dependent as well (Duckworth *et al*. 2001, Pérez-Rodríguez *et al*. 2006). Additionally, not only does testosterone suppress the immune system, but immune system activation suppresses testosterone production (Boonekamp *et al*. 2008, Ros *et al*. 2009, Greiner *et al*. 2010). This leads to a considerable flaw in the handicap argument. By nature, cheaters sever the link between their condition and [condition-dependent] trait expression, but the ICHH suggests that this cheating can be prevented by testosterone, another condition-dependent trait. Or put another way, the ICHH invokes one condition-dependent trait to regulate honesty of another condition-dependent trait.

Circumventing 'Testosterone'-mediated Immunosuppression

A fundamental aspect of a handicap mechanism is that the signal cannot be compromised by cheaters circumventing the handicap, but if the signal can be cheated, why not the handicap? Testosterone signalling pathways have the potential to be highly plastic in nature because the means by which they are neurally and physiologically regulated is highly complex and variable (Hau and Wingfield 2011).

How testosterone can impart a handicap that prevents cheating when the effect of testosterone on immune function is not obligatory is unclear. The variability of results from studies suggests a lability in the control of immunity (e.g., Roberts *et al*. 2004) or susceptibility to parasites (Fuxjager *et al*. 2011) by testosterone. For example, I have shown that at relatively low levels, testosterone may modulate immunity in female phalaropes, but not in males (Chapter 4). These results demonstrate that high levels of testosterone in male phalaropes are not necessarily immunosuppressive; the immunosuppressive cost of testosterone is not dose-dependent because low levels in females are immunosuppressive but high levels in males are not. Lability means that males can avoid the costs of immunosuppression caused by high levels of testosterone. Similarly, demonstrating a positive relationship between immune investment and plumage expression across species (Chapter 2) suggests that more colourful species are able to overcome the 'handicaps' predicted to be imposed through an ICHH-like mechanism in order to produce more elaborate plumage.

In broader terms, that individuals can develop insensitivities to the effects of testosterone is well documented (Lynn 2008), which again suggests that individuals can resist certain testosterone-mediated functions. Individual differences in hormone function can be governed through the modulation of transcription rates, hormone metabolism and receptor characteristics (Ball and Balthazart 2008). Therefore, as a rudimentary example, avoiding an immunocompetence handicap merely requires the reduction of androgen receptors on leukocytes relative to those responsible for signal expression. Indeed, it is expected that signalling systems evolve towards increased efficiency by shedding any unnecessary costs in their production, such as handicaps (Számadó 2011).

Other Arguments against Handicaps

Although often overlooked, the condition dependence of testosterone and the ability to avoid the immunosuppressive 'costs' of testosterone are important components of the argument against the ICHH. Together these points suggest that testosterone is a poor facilitator of a handicap because it is itself condition-dependent and because cheaters can circumvent the costs.

In a recent review of Zahavi's handicap principle, Számadó (2011) suggests that 'honesty' is maintained not through handicaps expressed at equilibrium, the main premise of the handicap principle which is supported by Zahavi and Zahavi (1997), Grafen (1990) and Maynard Smith (1991), but rather through costs to cheaters. As such, Számadó (2011) promotes what he terms the "fallacy of the handicap" (but see Grose 2011). Similarly, a model by Kelly and Alonzo (2010) showed that honesty can be maintained simply through a trade-off between current and future reproduction. Although their model specifically outlines a scenario where females assess the direct benefits in terms of parental ability from male signals, there is no reason to believe that the premise is not suitable for explaining any and all aspects of sexual signalling.

More recently, Emlen *et al*. (2012) proposed a mechanism for the expression of exaggerated sexual traits that is inherently reliable. The authors suggest that their mechanism, which explains trait expression through increased sensitivity to the insulin/insulin-like growth factor pathway, is not consistent with a handicap-type mechanism. Although Emlen *et al*.'s specific mechanism may not be directly appropriate for explaining plumage colouration, it promotes a valid alternative to handicaps in many systems where the ICHH is considered an important mechanism maintaining honesty (e.g., antlers: Malo *et al*. 2009). Together, these

theoretical and empirical arguments lend credence to the idea that handicaps are not necessary to maintain honest signals.

Why is Testosterone related to Immunocompetence if not through the ICHH?

If handicaps are not necessary to maintain honesty, why then does testosterone suppress immune function? By viewing testosterone in a life history context we have gained the insight that testosterone can tip species towards faster paced-lives (Evans 2010, Hau *et al*. 2010). Testosterone may function as a quarterback, tipping investment in favour of traits conferring high rates of current reproduction, such as breeding behaviours (i.e., aggressive territoriality) and secondary sexual traits (i.e., colourful plumage).

The most obvious functions of testosterone are directly related to reproduction, for example, spermatogenesis and the production of secondary sexual traits. But other functions of testosterone, such as immunosuppression, may be less obvious means to achieve efficient and effective reproduction. In mammals, testosterone levels are related to several aspects of energy metabolism. Specifically, high levels of testosterone reduce fat deposits, increase insulin sensitivity (see Emlen *et al*. 2012 for a direct link between insulin and reliable signalling) and improve mitochondrial processes (Traish *et al*. 2011). In birds, there is some evidence that testosterone can regulate energy reserves (Wingfield 1984, Ketterson *et al*. 1991, Deviche 1995), and in doing so could shunt resources from one function to another. As one mode of action, testosterone could exert metabolic actions in birds by regulating corticosterone levels (e.g., Breuner and Orchinik 2001, Ashley *et al*. 2009) because corticosterone plays an important role in energy metabolism (Remage-Healey and Romero 2001). Thus, testosterone could regulate energy flow to reproductive functions by sequestering resources from other important processes

either directly or indirectly, of which one outcome is immunosuppression (Owen-Ashley *et al*. 2004). Functioning in this manner, testosterone could liberate resources not otherwise available for reproduction. Therefore, through testosterone, males may gain the ability to inflate the expression of their condition-dependent traits by sequestering resources not ear marked for signal production. A tongue-in-cheek interpretation of this process is that rather than preventing cheating, testosterone promotes it. The degree of influence testosterone has should ultimately be a balance of the costs of sequestering resources from other functions and the reproductive advantages, that is to say a trade-off between current and future reproduction. Variation around the population mean value of the trait, which is centred on some optimal investment in the signal, has three sources: optimal investment that is determined by body condition, error in translating condition into signal intensity, and variation in investment that represents individual differences in pace-of-life. The latter two points imply that a certain amount of over-investment (i.e., cheating) should occur in any signalling system and that this variation is likely subject to natural selection.

Conclusion

In signalling theory, a handicap has a specific definition (Grafen 1990, Maynard Smith 1991, Zahavi and Zahavi 1997) and the ICHH was formulated in the spirit of this definition (Folstad and Karter 1992). It is conceivable that the original ICHH is contemporarily cited simply in the context of testosterone-mediated immunosuppression, breeding related immunosuppression, or reliable condition-dependent plumage expression aside from a handicap mechanism per se. Yet, the ICHH mechanism is commonly contrasted against other processes (Rolff 2002, Nunn *et al*. 2009, Számadó 2011) suggesting that the spirit of the handicap debate, aside from the debate

involving testosterone-mediated immunosuppression, is alive and well. Most notably, Emlen *et al*. (2012) provide a mechanistic explanation for inherent reliability of signal expression that contrasts with signal handicaps in general.

Models of inherent reliability recognize that traits are costly to produce, but that there are no handicaps above these initial costs. Studies demonstrating consequences to trait expression must then demonstrate that these costs are handicaps above the inherent costs of trait production. Although frequently cited to the contrary, it is questionable whether any evidence of a true immunocompetence handicap has been offered. Indeed, the ICHH is mostly supported simply by demonstrating 'honest' trait expression, or testosterone-mediated immunosuppression, but neither of these observations actually demonstrates a handicap at work and also are consistent with models of inherent reliability. Whereas much of the focus of the ICHH has been on whether testosterone is immunosuppressive, the library of work to date provides some clear evidence against the ICHH. In particular, the lack of consistent results (Roberts *et al*. 2004) demonstrates that immunosuppression is not a necessary consequence of high levels of testosterone which indirectly demonstrates the ability to avoid the handicap: testosterone can mediate the expression of certain traits but not others, including immunosuppression. In the current thesis, the relationships among sex, testosterone and immunity (Chapter 4) provide more direct evidence that testosterone-mediated immunosuppression can be circumvented when unnecessary. Additionally, the condition dependence of testosterone and the bidirectional feedback between testosterone production and the immune system demonstrates that testosterone is a poor candidate for providing a handicap. The challenge to resolving the debate between the ICHH and reliable signal expression is likely in defining a critical test to demonstrate either the existence of

a true handicap, or inherently reliable signal expression. Adapting the approach of Emlen *et al*.

(2012) to plumage traits could provide a model for doing so in birds.

Literature Cited

Araneo, B.A., T. Dowell, M. Diegel and R.A. Daynes. 1991. Dihydrotestosterone exerts a depressive influence on the production of interleukin-4 (IL-4), IL-5, and gamma-interferon, but not IL-2 by activated murine T cells. Blood 78:688-699.

Ashley, N.T., Q.R. Hays, G.E. Bentley, and J.C. Wingfield. 2009. Testosterone treatment diminishes sickness behavior in male songbirds. Horm Behav 56: 169-176.

Ball, G.F. and J. Balthazart. 2008. Individual variation and the endocrine regulation of behaviour and physiology in birds: a cellular/molecular perspective. Philos Trans R Soc Lond Ser B 363: 1699-1710.

Boonekamp, J.J., A.H.F. Ros and S. Verhulst. 2008. Immune activation suppresses plasma testosterone level: a meta-analysis. Ecol Lett 4: 741-744.

Braude, S., Z. Tang-Martinez and G.T. Taylor. 1999. Stress, testosterone, and the immunoredistribution hypothesis. Behav Ecol 10: 345-350.

Breuner, C.W., and M. Orchinik. 2001. Seasonal regulation of membrane and intracellular receptors in the house sparrow brain. J Neuroendocrinol 13: 412-420.

Buchanan, K.L. 2000. Stress and the evolution of condition-dependent signals. Trends Ecol Evol 15: 157-160.

Casto, J.M., V. Nolan Jr., and E.D. Ketterson. 2001. Steroid hormones and immune function: Experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). Am Nat 157: 408-420.

Deviche, P.E. 1995. Androgen regulation of avian premigratory hyperphagia and fattening: from eco-physiology to neuroendocrinology. Am Zool 35: 234-245.

Deviche, P. and L. Cortez. 2005. Androgen control of immunocompetence in the male house finch, *Carpodacus mexicanus* Müller. J Exper Biol 208: 1287-1295.

Duckworth, R.A., M.T. Mendonça and G.E. Hill. 2001. A condition-dependent link between testosterone and disease resistance in the house finch. Proc Biol Sci 268: 2467-2472.

Edler, R., W. Goymann, I. Schwabl and T.W.P. Friedl. 2011. Experimentally elevated testosterone levels enhance courtship behaviour and territoriality but depress acquired immune response in Red Bishops *Euplectes orix*. Ibis 153: 46-58.

Emlen, D.J., I.A. Warren, A. Johns, I. Dworkin and L.C. Lavine. 2012. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. Science 337: 860-864.

Evans, M.R. 2010. Why does testosterone influence morphology, behaviour and physiology? Open Ornithol J 3: 21-26.

Evans, M.R., A.R. Goldsmith, and S.R.A. Norris. 2000. The effects of testosterone on antibody production and plumage colouration in male house sparrows (*Passer domesticus*). Behav Ecol Sociobiol 47: 155-163.

Folstad, I., and A.J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. Am Nat 139: 603-622.

Fuxjager MJ, Foufopoulos J, Diaz-Uriarte R, Marler CA. 2011. Functionally opposing effects of testosterone on two different types of parasite: implications for the immunocompetence handicap hypothesis. Funct Ecol 25: 132-138.

Gil, D. and R. Culver. 2011. Male ornament size in a passerine predicts the inhibitory effect of testosterone on macrophage phagocytosis. Funct Ecol 25: 1278-1283.

Grafen, A. 1990. Biological signals as handicaps. J Theor Biol 144: 517-546.

Greiner, S., V. Stefanski, M. Dehnhard and C.C. Voight. 2010. Plasma testosterone levels decrease after activation of skin immune system in a free-ranging mammal. Gen Comp Endocrinol 168: 466-473.

Grose, J. 2011. Modelling and the fall and the rise of the handicap principle. Biol Phil 26: 677-696.

Hasselquist, D., J.A. Marsh, P.W. Sherman and J.C. Wingfield. 1999. Is avian humoral immunocompetence suppressed by testosterone? Behav Ecol Sociobiol 45: 167-175.

Hau, M. and J.C. Wingfield. 2011. Hormonally-regulated trade-offs: Evolutionary variability and phenotypic plasticity in testosterone signaling pathways. In: T. Flatt and A. Heyland (Ed.), Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits. Oxford University Press, NY.

Hau, M., R.E. Ricklefs, M. Wikelski, K.A. Lee and J.D. Brawn. 2010. Corticosterone, testosterone and life-history strategies of birds. Proc Biol Sci 277: 3203-3212.

Kelly, N.B., and S.H. Alonzo. 2010. Does a trade-off between current reproductive success and survival affect the honesty of male signalling in species with male parental care? J Evol Biol 23: 2461-2473.

Ketterson, E.D., V. Nolan, L. Wolf, C. Ziegenfus, A.M. Dufty, G.F. Ball and T.S. Johnsen. 1991. Testosterone and avian life histories. The effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos. Horm Behav 25: 489–503.

Liva, S.M., and R.R. Voskuhl. 2001. Testosterone acts directly on $CD4^+$ T lymphocytes to increase IL-10 production. J Immunol 167: 2060-2067.

Lynn, S.E. 2008. Behavioral insensitivity to testosterone: Why and how does testosterone alter paternal and aggressive behavior in some avian species but not in others? Gen Comp Endocrinol 157: 233-240.

Malo, A.F., E.R.S. Roldan, J.J. Garde, A.J. Soler, J. Vicente, C. Gortazar and M. Gomendio. 2009. What does testosterone do for red deer? Proc Biol Sci 276: 971-980.

Mateos, C. 2005. The subordination stress paradigm and the relation between testosterone and corticosterone in male ring-necked pheasants. Anim Behav 69: 249-255.

Maynard Smith, J. 1991. Honest signalling: the Philip Sidney Game. Anim Behav 42: 1034-1035.

Mougeot, F., S.M. Redpath, S.B. Piertney and P.J. Hudson. 2005. Separating behavioral and physiological mechanisms in testosterone-mediated trade-offs. Am Nat 166: 158-168.

Nunn, C.L., P. Lindenfors, E.R. Pursall and J. Rolff. 2009. On sexual dimorphism in immune function. Phil Trans R Soc B 364: 61-69.

Okado, H., J. Banchereau and M.T. Lotze. 2003. Interleukin-4. In: A.W. Thomson and M.T. Lotze (Ed.), The Cytokine Handbook, 4th ed. Academic Press, New York.

Oliveira, R.F. 2004. Social modulation of androgens in vertebrates: mechanisms and function. Adv Study Behav 34: 165-239.

Owen-Ashley, N.T., D. Hasselquist, and J.C Wingfield. 2004. Androgens and the immunocompetence handicap hypothesis: Unraveling direct and indirect pathways of immunosuppression in Song Sparrows. Am Nat 164: 490-505.

Pérez-Rodríguez, L., J. Blas, J. Viñuela, T.A. Marchant and G.R. Bortolotti. 2006. Condition and androgen levels: are condition-dependent and testosterone-mediated traits two sides of the same coin? Anim Behav 72: 92-103.

Poiani, A., A.R. Goldsmith and M.R. Evans. 2000. Ectoparasites of house sparrows (*Passer domesticus*): an experimental test of the immunocompetence handicap hypothesis and a new model. Behav Ecol Sociobiol 47: 230-242.

Råberg, L., M. Grahn, D. Hasselquist, and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. Proc Biol Sci 265: 1637-1641.

Rantala, M.J., A. Vainikka and R. Kortet. 2003. The role of juvenile hormone in immune function and pheromone production trade-offs: a test of the immunocompetence handicap principle. Proc Biol Sci 270: 2257-2261.

Remage-Healey, L. and L.M. Romero. 2001. Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. Am J Physiol Regul Integr Comp Physiol 281: R994– R1003.

Roberts, M.L., K.L. Buchanan, and M.R. Evans. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. Anim Behav 68: 227-239.

Roberts, M.L., K.L. Buchanan, D. Hasselquist, and M.R. Evans. 2007. Effects of testosterone and corticosterone on immunocompetence in the zebra finch. Horm Behav 51: 126-134.

Roberts, M.L., K.L. Buchanan, M.R. Evans, R.H. Marin, and D.G. Satterlee. 2009. The effects of testosterone on immune fuction in quail selected for divergent plasma corticosterone response. J Exp Biol 212: 3125-3131.

Rolff, J. 2002. Bateman's principle and immunity. Proc Biol Sci 269: 867-872.

Romero, L.M., and J.C. Wingfield. 1998. Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's White-crowned sparrows (*Zonotrichia leucophrys gambelii*). Comp Biochem Physiol 119C: 31-36.

Ros, A.F.H., M. Correia, J.C. Wingfield and R.F. Oliviera. 2009. Mounting an immune response correlates with decreased androgen levels in male peafowl, *Pavo cristatus*. J Ethol 27: 209-214.

Sapolsky, R.M., M. Romero, and A.U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparatory actions. Endocr Rev 21: 55-89.

Senar, J. C. 2006 Color displays as intrasexual signals of aggression. In G. E. Hill & K. J. McGraw (Ed.), Bird coloration: function and evolution, vol. 2. Harvard University Press, Cambridge, MA.

Shelby, J.A., R. Madewell and A.P. Moczek. 2007. Juvenile hormone mediates sexual dimorphism in horned beetles. J Exp Zool B Mol Dev Evol 308: 417-427.

Shingleton, A.W., and W.A. Frankino. 2013. New perspectives on the evolution of exaggerated traits. Bioessays 35: 100-107.

Shtrichman, R., and C.E. Samuel. 2001. The role of gamma interferon in antimicrobial immunity. Curr Opin Microbiol 4: 251-259.

Slater, C.H., M.S. Fitzpatrick and C.B. Schreck. 1995. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen Comp Endocrinol 100: 218-225.

Számadó, S. 2011. The cost of honesty and the fallacy of the handicap principle. Anim Behav 81: 310.

Takatsu, K, T. Kouro and Y. Nagai. 2009. Interleukin 5 in the link between the innate and acquired immune response. Adv Immunol 101: 191-236.

Traish, A.M., B. Abdallah and G. Yu. 2011. Androgen deficiency and mitochondrial dysfunction: implications for fatigue, muscle dysfunction, insulin resistance, diabetes, and cardiovascular disease. Horm Mol Biol Clin Invest 8: 431-444.

Wingfield, J.C. 1984. Environmental and endocrine control of reproduction in the song sparrow, *Melospiza melodia*. II. Agonistic interactions as environmental information stimulating secretion of testosterone. Gen Comp Endocrinol 56: 417–424.

Young, H.A., and K.J. Hardy. 1995. Role of interferon-gamma in immune cell regulation. J Leukoc Biol 58: 373-381.

Zahavi, A. 1975. Mate selection: a selection for a handicap. J Theor Biol 53: 205-214.

Zahavi, A., and Zahavi, A. 1997. The Handicap Principle. New York: Oxford University Press.